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TITLE OF THESIS....STUDIES ON THE A PROTEIN OF BACTERIOPHAGE R17.

UNIVERSITY....UNIVERSITY OF ALBERTA, EDMONTON

DEGREE FOR WHICH THESIS WAS PRESENTED....MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED....1976

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THE UNIVERSITY OF ALBERTA

STUDIES ON THE A PROTEIN OF BACTERIOPHAGE R17

by

(C) SHIRLEY M. REYNOLDS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Studies on the A Protein of Bacteriophage R17", submitted by Shirley M. Reynolds in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry.

Wm. Paranchych
Supervisor

Mike Longley

Doug Scraba

Date....7 July.....1976

ABSTRACT

The aim of the study to be described in this thesis was to establish the location of the A protein in bacteriophage R17 and to isolate an infectious A protein-RNA complex from the virus.

Neutralization of the infectivity of phage R17 by antibodies prepared against highly purified A protein, provides direct evidence that this protein is present on the surface of the virus particle. This location is confirmed by the finding that lactoperoxidase catalyzes the iodination of the A protein as well as the coat protein monomers of intact phage particles, which have been purified by non-disruptive means.

Cross-linking studies using bifunctional reagents were initiated in the hope of establishing the topographical location of the A protein in the phage capsid. However, these studies yielded no information regarding the neighborhood of the A protein, but the formation of coat protein dimers was observed and this is discussed in relation to the recent models of the structure of RNA phages.

An A protein-RNA complex which is infectious to intact E. coli cells was isolated from the coprecipitate of A protein and RNA which results from acetic acid treatment of intact phage particles. Isolation of the complex was achieved by equilibrium centrifugation in Cs_2SO_4. This complex is disrupted by the perturbants, ethylene glycol and dimethyl sulfoxide which suggests that both hydrogen and hydrophobic bonds are required in its maintenance. That similar bonding types also exist in the intact phage particle is suggested by the finding that the A protein is dissociated from the RNA upon treating the particles with 8 M urea.

ACKNOWLEDGEMENTS

First and foremost, I wish to express my appreciation to Dr. W. Paranchych for his invaluable advice and direction throughout the course of these studies.

My gratitude is also extended to Mrs. M. Bangen and Mrs. M. Tichy, who assisted with the preparation of the phage. I also wish to thank Mrs. P. Finsten who typed the first chapter of this thesis, and Miss C. Hicks who processed the photographs.

Finally, I am grateful to the Department of Biochemistry which provided financial aid in the form of teaching assistantships.

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LIST OF ABBREVIATIONS

rpm	revolutions per minute
cpm	counts per minute
S (following a number)	sedimentation constant in Svedberg units (10^{-13} sec)
A_{412} (A_{280})	light absorbancy of a solution in a 1 cm light path at 412 nm (280 nm)
Ci	Curie; 2.2×10^{12} disintegrations per minute
mA	milliamperes
PFU	plaque forming units
DNA	deoxyribonucleate
RNA	ribonucleate
RNase	ribonuclease
A or A_{OH}	adenosine
C	cytidine
AMP	adenosine monophosphate
GMP	guanosine monophosphate
pppGp or G4P	guanosine 5'-tri, 2',3'-monophosphate
SSC	standard saline citrate 0.15 M NaCl, 0.015 M sodium citrate
TMM	Tris(hydroxymethyl) aminomethane maleic acid minimal salts medium
Tris	Tris(hydroxymethyl) aminomethane
EDTA	ethylenediaminetetraacetate
EtOH	ethanol
MeOH	methanol
PEG	polyethylene glycol 6000
NaDS	sodium dextran sulfate

TEMED	N,N,N',N'-tetramethylethylenediamine
SDS	sodium dodecyl sulfate
MMB	methyl 4-mercaptobutyrimidate hydrochloride
DMS	dimethyl suberimidate dihydrochloride
DMSO	dimethyl sulfoxide
G-HCl	guanidine hydrochloride
TCA	trichloroacetic acid
DNP	dinitrophenol
DEAE cellulose	diethylaminoethyl cellulose
IgG	immunoglobulin

All temperatures are expressed in degrees Centrigade.

CHAPTER I

INTRODUCTION

Since the discovery (Steitz, 1968a) that the product of the A cistron (viz. the A protein also called the maturation protein) of RNA bacteriophages is a structural component of the virus particle where it is present in only one copy, a great deal of work has been done in an attempt to characterize the protein and to obtain information regarding its location in the particle and its function during infection. While the A protein has been shown to be essential for correct assembly and for infectivity, the exact role it plays in these processes has not yet been clarified.

The purpose of the present study was to determine the location of the A protein in bacteriophage R17 and to investigate its role in infectivity. The following will be a brief resume of those aspects of the phage system relevant to this study.

A. Phage Structure

Since the isolation of bacteriophage f2 by Loeb and Zinder (1961), many RNA coliphages have been isolated from sewage effluents throughout the world. Structurally, these phages are very simple being composed of a single-stranded RNA genome of about 3,500 nucleotides (Fiers et al., 1976) with a molecular weight of 1.1×10^6 (Sinha et al., 1965), which is enclosed by a capsid containing 180 copies of the coat protein (MW = 13,750) and one copy of the A protein (MW = 40,000). Despite the different geographical locations of their origins, these phages are very similar and they can be divided into three groups on the basis of the chemical and immunological properties of their coat proteins

(Watanabe et al., 1967; Nishihara et al., 1969). The group I phages (whose members include f2, R17, fr, MS2, M12 and ZR) have been most studied; the amino acid sequences of many of their coat proteins have been determined and it has been found that they differ from one another only by a few amino acid substitutions (Weber and Konigsberg, 1967; Weber, 1967; Wittman-Liebold and Wittman, 1967; Nishihara et al., 1970). Apart from Qβ (Group III) for which the coat protein sequence has been worked out (Maita and Konigsberg, 1971), only the amino acid compositions of the coat proteins of the phages in Groups II and III are known. In general, the amino acid composition of the coat protein of a member of a group is very similar to that of another member of the same group and properties determined for one member can usually be applied to the others.

The feature of the RNA phages which has made them such important objects of study is the small size of their genomes. Complementation analyses and physiological analyses of the amber and temperature-sensitive mutants of the phages have established the existence of three cistrons (Horiuchi et al., 1966; Gussin, 1966; Horiuchi and Matsuhashi, 1970) and the order of the genes on the genome has been deduced by Jeppeson et al. (1970) to be 5'-A-Coat-Replicase-3'. This deduction has been corroborated by the correspondence of the nucleotide sequence of the coat cistron (Min Jou et al., 1972) and the A cistron (Fiers et al., 1975) to the amino acid sequence of the coat protein (Lin et al., 1967; Vandekerckhove et al., 1969) and the A protein (Fiers et al., 1975). Recently, the sequence of the replicase gene was reported (Fiers et al., 1976) so that the entire primary structure of the genome has now been established.

The RNA of these phages is present in compact form as indicated by the low radius of gyration and the high sedimentation coefficient (26S in 0.1 M NaCl) (Gesteland and Boedtker, 1964). A helix content of about 70% was estimated for the isolated RNA and of about 80% for the RNA as packed into the capsid (Mitra et al., 1963; Boedtker, 1967). In 0.01 M NaCl (Gesteland and Boedtker, 1964) and in formaldehyde (Boedtker, 1968; Spahr et al., 1969) the RNA extends to a more linear form and has a much lower sedimentation coefficient. The organization of the RNA inside the capsid is still unclear. Low-angle X-ray scattering studies have shown that the phage particle has a mean outer radius of 131.7 Å (Zipper, 1971) and consists of an outer protein shell of width 30 Å (Fischbach et al., 1965) and a less dense inner core at the center of which is a hole of radius 15 Å. The core contains 80% of the RNA; the remaining 20% is considered to penetrate into the shell most probably intercalated between the protein sub-units. Hohn (1969) suggested that some of the basic amino acids of the coat protein may interact with the phosphate groups of the RNA. If all the basic residues were involved in such binding, two-thirds of the RNA would be bound while the rest could form a core with the phosphate groups neutralized by counter-ions such as Mg^{++} or spermidine. The latter is an organic cation and phage RNA isolated with phenol and sodium dodecyl sulfate has been shown to contain 70 to 90 moles of it per mole (Fukuma and Cohen, 1975). Hohn's concept was extended by Matthews and Cole (1972) who proposed a structure in which the acidic amino acids are on the outside interacting with solvent while the hydrophobic amino acids lie between the negatively and positively charged groups.

Studies of electron micrographs have shown that these phages have overall icosahedral symmetry but the detailed topography of the virus remains to be clarified. Several models have been proposed in an attempt to account for the arrangement of 180 identical subunits on an icosahedron. Such an arrangement cannot be perfectly symmetrical and the subunits are only in "quasi-equivalent" conditions (Caspar and Klug, 1962), which means that there are variations in the lengths and angles of bonds between the subunits. Therefore, it was initially predicted that such icosahedral structures would be stable only after they have been completed and that their final structure would be determined by a morphopoietic factor such as nucleic acid. However, it was subsequently demonstrated (Matthews and Cole, 1972) that under carefully controlled conditions, coat protein monomers can undergo assembly to form stable empty capsids. Vasquez et al. (1966) studied the morphology of negatively-stained R17 particles by electron microscopy and concluded that the capsid proteins are arranged as 20 hexamers and 12 pentamers. Subsequent studies by Klug et al. (1966) and Hohn and Hohn (1970) led to the formulation of a 60 capsomere model. An alternative mode of ascertaining the structure, viz., by the characterization of the intermediates released by controlled disassembly of the particles in guanidine-HCl (O'Callaghan et al., 1973) led to the proposal that the capsid comprises 20 nonagons. Such an arrangement violates the principle of quasi-equivalence and hence a revised model has been proposed (Dunker and Paranchych, 1975) in which the protein subunits are arranged in dimer formation with rings of five about the five-fold axis and rings of six about the three-fold axis. The latter model has been further substantiated by three-dimensional image reconstructions

from electron micrographs of phage particles (Crowther et al., 1975).

B. A protein and its involvement in phage infection.

The RNA bacteriophages specifically infect Hfr, F^+ or F' strains of E. coli all of which possess a piece of extrachromosomal DNA, i.e., a plasmid which can code for the production of F pili. The latter, which were first observed in the electron microscope by Crawford and Gesteland (1964), are long, thin surface appendages to the sides of which large numbers of RNA phage can attach. To date, little is known about the mechanism by which these structures mediate the penetration of the phage genome into the host cell. Negatively-stained pili show an axial hole which is about 2.5 nm in diameter (Brinton et al., 1964) and this led to the proposal that F pili may serve as hollow tubes for nucleic acid transport. Several other models have been proposed since then. Marvin and Hohn (1969) and Curtiss (1969) suggest that attachment of the phage to the side of the pilus triggers a retraction of the latter through a process of sequential depolymerization of pili subunits within or at the cell membrane. This process would lead to the arrival of the phage at the base of the pilus where the RNA could be injected into the cell. More recently, Brinton (1971) modified his earlier model by making the assumption that F pili consist of two parallel protein filaments which either permit passage of the nucleic acid ("conduction") or which move relative to one another and so transfer the molecule in a "conveyer belt" mechanism.

That the A cistron product is in some way required for the assembly of infectious particles was first suggested by the fact that phage-like particles which are both structurally defective and non-infectious are produced upon infection of suppressor-negative bacteria with phage

containing an amber mutation in the A cistron (Lodish et al., 1965; Heisenberg and Blessing, 1965; Argetsinger and Gussin, 1966). The defective particles produced in such a system appear normal in the electron microscope but they cannot attach to host cells and while their RNA is intact and infectious if grown on an RNase I^- host (Heisenberg, 1966), part of the RNA is degraded when the particles are exposed to nucleolytic attack. The wild-type phage in comparison is stable to RNase. The digested defective particles - light defective particles - band as a broad peak in CsCl density gradients at lower density than the intact phage and corresponding to an average RNA content of two-thirds that of the original. The sedimentation coefficients of defective and light defective particles are 69S and 74S respectively as compared with 80S for the wild-type phage. The lower sedimentation of the particle lacking only A protein is probably due to higher friction because of a protruding piece of RNA since RNase treatment results in an increase in the rate of sedimentation even though the molecular weight is decreased. Another indication that RNA protrudes from the defective particles is that they react with anti-RNA antiserum while wild-type phage do not (Heisenberg, 1967). From all of these studies, it was concluded that the class A amber mutants are structurally defective. Lodish et al. (1965) suggested that the structural aberration might be due to the lack of an enzyme specified by gene A and responsible for assembly of the phage particles. Argetsinger and Gussin (1966) suggested that the A protein is a structural protein and functions as an attachment organelle. Since the particles produced by the several class A mutants of R17 studied vary in the extent to which their RNA is available to nucleo-

lytic degradation, the mutants must differ structurally from one another. A structurally incomplete enzyme would not be expected to function catalytically and hence the former suggestion does not seem feasible. On the other hand, it is easy to envision that an incomplete structural protein, though unable to function in attachment, might be able to associate with the virus particle and exert some control over the extent of degradation of the RNA.

Steitz (1968a) confirmed that the A protein is indeed a structural component of the phage by exploiting the fact that the A protein can be specifically labeled with histidine since it contains this amino acid (Vinuela et al., 1967) while the coat protein does not (Weber, 1967). Electrophoretic examination of ^{3}H histidine-labeled R17 using SDS-polyacrylamide gels showed the presence of a polypeptide (MW 35,000-40,000) which coelectrophoresed with a phage-specific protein isolated from actinomycin-treated infected spheroplasts. Also, no protein of this mobility was observed in non-infective gene A amber mutants of R17 grown on suppressor-negative hosts.

The purification of A protein from the intact virion is complicated by the insolubility of the phage structural proteins in non-denaturing solvents and by the low molar ratio of A protein to coat protein. Despite these difficulties, Steitz (1968b) developed a purification procedure by following the fate of the histidine-labeled component of R17. Ion-exchange chromatography on phosphocellulose, subsequent to dissociation in 6 M guanidine-HCl and dialysis into 8 M urea, permitted the separation of A protein from the coat and the RNA in a single step. All of the RNA and 98% of the coat protein appear in the flow-through volume. The A protein adheres to the column, is eluted with a linear

NaCl gradient and is dissolved in buffer containing 8 M urea.

Osborn et al. (1970) describe a procedure which yields sufficient amounts of A protein for chemical studies. This method is simpler than that described by Steitz and permits nearly quantitative recovery of pure A protein. The method makes use of the fact that the coat protein is soluble in 66% acetic acid while the A protein and RNA form a precipitate. Upon treating the latter with potassium dodecylsulfate, the RNA is removed, leaving highly purified A protein from which the residual dodecylsulfate is removed by washing with acetone.

Amino acid analysis was performed on the purified A protein by Steitz (1968b) and the amino acid composition was compared with that of the coat protein. The most notable differences are the absence of histidine from the coat and the presence of this amino acid in the A protein. Also, the A protein has a higher content of arginine. Five residues of histidine occur per 37,500 daltons of A protein, and isotopic methods reveal an average of 4 histidine residues per wild-type phage particle. Therefore, there is only approximately one A protein molecule per bacteriophage particle. The sequence of the A protein is almost complete (Fiers et al., 1975) and a notable feature is the clustering of hydrophobic amino acids which might explain the tendency of this protein to adhere to dialysis membrane and other surfaces even when dissolved in denaturing solvents (Roberts and Steitz, 1967; Steitz, 1968b; Valentine et al., 1969; Hohn and Hohn, 1970). No regions of extensive homology were observed between the coat and the A protein.

Studies on the interaction of phage with F pili are complicated by the fact that RNA phage preparations are heterogeneous with respect

to the attachment function. The attachment assay is based on the fact that phage which are bound to F pili are retained on a membrane filter whereas unadsorbed phage pass straight through. Only 10% of the phage population are infectious (Class I). The remainder of the phage can be divided into two categories. Class II particles (80% of the population) adsorb much less effeciently than Class I particles and although the A protein is transferred into the host cell after interacting with it, little or no RNA is released from the phage (Paranchych et al., 1971). It has been suggested that the defect in Class II particles is due to a dissociation of an A protein-RNA complex, although of course, it may be due simply to a break in the RNA chain at or near the region of contact with the A protein. Ejection of the A protein from Class II particles renders them sensitive to RNase suggesting that the RNA becomes exposed when the A protein is removed. The remaining 10% of the population (Class III) are non-infectious and have been shown to lack the A protein but they are completely resistant to RNase (Krahn and Paranchych, 1971).

Class III particles may possibly be an artefact resulting from the use of CsCl density gradient centrifugation in the purification procedure. It has been shown by Verbraeken and Fiers (1972) that 50% of the phage particles lose their A protein upon centrifugation in a cesium chloride gradient. The resulting particles resemble the Class III particles in that they are non-infectious and resistant to RNase. The reason for such a great loss of A protein from particles in CsCl observed by Verbraeken and Fiers may be due to the different purification procedure employed. While the latter workers used ammonium sulfate precipitation followed by freon extractions, Krahn and

Paranchych (1971) used a polyethylene glycol-sodium dextran sulfate two-phase system. The lability of the A protein on the phage particle is further emphasized by the finding that this protein is removed from intact particles at concentrations of sodium dodecyl sulfate insufficient to solubilize the coat protein subunits (Knolle, 1972).

Reconstitution of phage has led to a greater understanding of the role of the A protein. The first attempts to assemble particles in vitro were carried out by Hohn (1967) and Sugiyama (1967) using coat protein isolated by the acetic acid method and RNA isolated by phenolisation. The main products were particles which resembled wild-type phage as judged by their appearance under the electron microscope and which in the absence of RNase had the same buoyant density in CsCl (1.43 g/cc) and contained a normal amount of RNA. However, the infectivity was very low (10^{-7}-10^{-8}), the sedimentation constant was only 70S and part of the RNA was sensitive to RNase. Hence, these particles resemble the A amber mutants. Possession of the purified A protein enabled Roberts and Steitz (1967) to carry out reconstitution of phage R17 using all the components. They showed that a significant synthesis of infective particles occurs only when A protein is included in the assembly mixture and they suggest that the low infectivity observed in the particles assembled from RNA and coat protein alone is due to residual A protein in the coat protein preparation. Similarly in Qβ, a linear response of PFUs as a function of added A protein was observed (Hung and Overby, 1969). However, the overall yield in infectivity is low in both the R17 and Qβ systems - 2×10^{-6} RNA molecules per input RNA or less are converted into infectious particles. This is in sharp contrast to the situation for TMV and the small ico-

sahedral plant viruses where the infectivity restored in *in vitro* reconstitution experiments is of the same order of magnitude as the infectivity of the *in vivo* produced virus (Bancroft, 1970). The success of the latter experiments, however, is probably due to the easier mode of infection of plant viruses which are believed to enter only damaged cells.

Kaerner (1970) suggests a reason for the low yield of infectious phage in reconstitution experiments. He found that if infected *E. coli* cells are starved of histidine 12 min after infection, i.e., just prior to initiation of synthesis of the A protein, non-infectious particles are formed. These particles resemble the defective phage produced by Class A amber mutants as judged by their sedimentation velocity, CsCl buoyant density and RNase sensitivity. Such defective particles are not converted to infectious phage if synthesis of the A protein is resumed in the host cells. Kaerner therefore suggests that the binding of A protein to RNA is an early event in the phage assembly process possibly governing the correct aggregation of the main coat protein subunits which leads to the formation of intact infective viruses. That the A protein may have a primary role in assembly is further suggested by the isolation from newly-infected cells of an early assembly intermediate consisting of a ribonucleo-protein which is rich in A protein (Bonner, 1974; Cramer and Sinsheimer, 1971; Richelson and Nathans, 1967). Also, the fact that the bulk of the A protein is synthesized prior to synthesis of the major part of the coat protein (Vinuela *et al.*, 1967) lends support to the theory that the A protein is required in the early stages of assembly.

However, the relative time at which the A protein enters the

assembly process does not allow any prediction about its final location in the particle. The different functions of the phage attributed to presence of the A protein, viz., protection of the RNA from nucleolytic degradation, adsorption to the bacterial pili and release of RNA during the infectious process have given rise to different models for the location of the A protein on the virion. According to the "core" model, the A protein is located inside the capsid where it can stabilize the RNA and at the same time confer infectivity by determining the overall conformation of the particle. According to the "tail" model, on the other hand, the A protein is located on the phage surface where it is thought to have the primary functions of adsorbing the phage particle to the host and triggering release of the phage RNA (Argetsinger and Gussin, 1966; Steitz, 1968b). Evidence in support of the latter model has been obtained by Curtiss and Krueger (1974) who showed that the A protein, isolated from MS2 particles which have been conjugated with dinitrophenol (DNP), contains covalently attached DNP and who further showed that lactoperoxidase catalyzes the iodination of the A protein as well as the coat protein monomers in intact MS2 particles.

The position of the A protein in the particle cannot be visualized in the electron microscope. Although the three-dimensional image reconstructions from electron micrographs of phage particles (discussed above) give some idea as to the distribution of the capsid protein, the contribution of the A protein is lost when the data are icosahedrally averaged (Crowther et al., 1975). However, in their structural model for R17, Dunker and Paranchych (1975) propose that the A protein could protrude through an opening at either the three-fold or the

five-fold axis of the icosahedral particle.

Several lines of evidence suggest the importance of an A protein-RNA complex during the process of infection. Krahn et al.(1972) found that the interaction of R17 particles with F piliated bacteria at 37° leads to the cleavage of the A protein (MW 39,000) into two smaller components (of molecular weights 24,000 and 15,000) which are transferred into the cell along with the phage RNA. A similar co-transfer of A protein and RNA into the host cell during infection with MS2 was observed by Kozak and Nathans (1971). The fact that the A protein and RNA are injected into the cell in approximately equimolar amounts and that their kinetics of penetration are similar (Krahn et al., 1972) suggests that these components may be transferred into the host bacterium as a complex. That an A protein-RNA complex may exist in intact phage is suggested by the findings of Oriel (1969) that the A protein is associated with RNA fragments released from whole phage following alkaline or heat treatment. Furthermore, the coprecipitate of A protein and RNA produced by treating intact phage at 4° with 66% acetic acid (Osborn et al., 1970) has been shown to contain an A protein-RNA complex which sediments at the same rate as free RNA in a sucrose gradient and which, unlike free RNA, is infectious to intact E. coli cells (Leipold and Hofschneider, 1975). A similar infectious A protein-RNA complex was prepared in vitro by the addition of highly purified A protein to RNA in a reaction mixture containing 5 M guanidine-HCl and the subsequent removal of the latter by dialysis (Shiba and Miyake, 1975). If such an A protein-RNA complex is indeed present in intact particles, then presumably the A protein is associated with the RNA in a unique fashion, possibly with the portion which is

injected first during the infectious process. Lodish (1968) proposed that the 5'-terminus of the RNA is injected first since defective particles contain an intact 3'-end but are lacking a 5'-end. However, the recent findings of Wong and Paranchych (1976a) have not corroborated Lodish's proposal. Following limited cleavage of R17 RNA *in situ* with ascorbate-Cu^{++} treatment, they permitted interaction of the treated phage with a bacterial host. The infection was terminated when it was observed that the A protein together with an RNA fragment had entered the host cell. Examination of the RNA extracted from the partially empty capsids for the presence of the 5'-(pppGp) and the 3'-adenosine (A) termini revealed that the G4P/GMP ratio had increased while the A/AMP ratio had markedly decreased. These findings led Wong and Paranchych to propose that the 3'-end of the phage genome acts as the pilot end during the RNA penetration process. Therefore, it seems feasible that a site at or near the 3'-terminus may well be the primary recognition site for the A protein. Presumably, the coat protein then associates around this A protein-RNA complex in such a way that the A protein is exposed on the surface of the resulting icosahedral particle. Such a location would enable the A protein to carry out a dual role in the infectious process, firstly as an attachment organelle by specific interaction with the F pilus and secondly as a pilot protein which in some as yet unknown manner, facilitates the entry of the RNA into the host bacterium.

The present investigation was aimed at obtaining further information regarding the role of the A protein in the R17 infectious process. Although considerable indirect evidence was already available suggesting that the A protein occupies a surface location on the

virion and that it is physically linked to the phage RNA, no direct evidence to support either of these ideas was available. Studies were thus initiated in this laboratory to determine whether the A protein in whole phage can be inactivated by anti-A protein antibodies and/or whether it can be iodinated by lactoperoxidase. Studies were also carried out to examine the effects of various cross-linking reagents on the capsid proteins. Attempts to isolate the A protein-RNA complex were greatly aided by the report of Leipold and Hofschneider (1975), which showed that a 66% acetic acid precipitate of phage RNA and A protein can be solubilized in a Tris-EDTA buffer, and that this soluble material contains small quantities of an infectious complex of RNA and A protein. As described in what follows, these studies provide overwhelming evidence that the A protein is a surface protein and that it is physically connected to the phage RNA.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Bacteriophage and Host Bacterium

The RNA bacteriophage, R17, originally isolated by Paranchych and Graham (1962) and a host bacterium, Escherichia coli (E. coli) WP156 (Hfr met$^-$ T_1^r Sm^s) were used throughout the studies described in this thesis.

The bacteria were maintained on hard agar plates for routine use. Permanent stocks were stored on hard agar slants in wax-sealed vials. Cell cultures were started by transferring a single colony from an agar plate to either 10 ml of minimal medium which was shaken in a 37° water bath overnight or 5 ml of L Broth which was grown statically in a 37° incubator overnight.

2. Bacterial Culture Media

(a) Supplemented Tris(hydroxymethyl) aminomethane maleic acid minimal salts (TMM) medium

The basic TMM salts solution contained the following components in moles per liter; Tris, 0.05; maleic acid, 0.05; NaCl, 0.043; KCl, 0.027; NH_4Cl, 0.019; Na_2HPO_4, 0.001; Na_2SO_4, 0.001.

After dissolving the above in distilled water, the pH was adjusted to 7.3 with concentrated NaOH and the solution autoclaved at 126° for 15 min under a steam pressure of 20 lbs/in^2.

In order to support the growth of E. coli WP156, this salts

solution was supplemented by adding sterile nutrient solutions in the following proportions:

Basic TMM salts solution	910 ml
50% (w/v) glucose	10 ml
0.25% (w/v) L-methionine	10 ml
0.5 M $MgCl_2$	10 ml
20 mg % d-biotin	50 ml

This will be referred to as "supplemented TMM medium" throughout the course of this thesis.

(b) L Broth

Tryptone (Difco)	10 g/l
Yeast extract (Difco)	5 g/l

Autoclaved solutions had a pH of 7.2 - 7.3

(c) Hard Agar

Trypticase soy broth	30 g/l
Bacto-Agar (Difco)	15 g/l

After dissolving, the solution was autoclaved and dispensed while warm into disposable Petri dishes.

(d) Top Agar

Top agar was prepared as above except that the final agar concentration was 11 g/l. Sterile top agar was stored in 50 ml volumes until used. In preparation for plaque assays, the agar was melted in a boiling water bath, dispensed in 2 ml aliquots into sterile culture tubes and maintained in the liquid state until used by incubating at 50° in a Temp-Blok (Lab-Line Instruments, Inc.).

3. Bacteria and Phage Diluent

All dilutions of bacteria or phage were made with a sterile solution composed of 0.9% (w/v) NaCl, 5 mM $MgCl_2$ and 5 mg % bovine serum albumin. Diluent was dispensed in 10 ml volumes into sterile 20 mm dilution tubes and stored at 4°. These solutions were prewarmed to room temperature before use.

4. Chemicals, Enzymes and Reagents

Unlabeled amino acids, ribonucleosides, sodium dextran sulfate 500-S, lysozyme (egg-white), pancreatic ribonuclease (5 x recrystallized), lactoperoxidase (A_{412}/A_{280} = 0.75) and bovine serum albumin were purchased from Sigma Chemical Co. Urea (ultra-pure), guanidine hydrochloride (ultra-pure), sucrose (ultra-pure, RNase-free) and Coomassie Brilliant Blue were obtained from Schwarz/Mann. Sodium dodecyl sulfate was from Matheson, Coleman and Bell and the agarose (Indubiose A37) from L'industrie Biologique Francaise. The ethylene glycol, acetic acid and sodium iodide were 'Baker Analyzed' Reagents and the dimethyl sulfoxide, hydrogen peroxide and bromophenol blue (0.04%) were Certified Fisher Reagents. Polyethylene glycol 6000 was supplied by J.T. Baker and Co., as was the acetone ('Baker Instra-Analyzed' Reagent). The cesium chloride (Sequanal Grade) was obtained from Pierce and the cesium sulfate (99.9%) from Apache Chemicals. The dimethyl suberimidate dihydrochloride was purchased from Aldrich, the methyl 4-mercaptobutyrimidate from ICN and the glutaraldehyde (8%, pH 5, under nitrogen) from Polysciences. The Amylose Azure (B Grade) was supplied by Calbiochem. N,N,N',N'-tetramethylethylenediamine (TEMED), acrylamide and N,N'-bis-methylene (bis) acrylamide were obtained from Eastman Kodak. The latter two were recrystallized before use according to

the procedure published by Loening (1967). Column Coat was purchased from Canalco and the NCS reagent employed for solubilizing polyacrylamide gel slices was from Nuclear Chicago.

5. Radioactive Materials

Radioactive precursors for the preparation of labeled phage R17 were obtained from the following sources: ^{32}P (inorganic phosphate, carrier free) from New England Nuclear; Na ^{125}I (carrier free) and ^{3}H-2,5-histidine (45 Ci/mmole) from Amersham/Searle.

B. Growth of Bacteria

Experimental cultures were grown at 37° in a rotary-shaking water bath from a 1/50 dilution of an overnight E. coli culture. In order to achieve maximum aeration and avoid excessive breakage of F pili, shallow cultures (generally 20% of the flask volume) were shaken at a speed of 125 rpm in baffled culture culture flasks (Bellco Glass Co.). Under these conditions, cultures generally reached a cell density of 4×10^8 cells/ml about 4 hrs after inoculation if grown in a minimal salts medium, and about 2 1/2 hrs for cells growing in L Broth.

The density of viable E. coli cells in TMM media was determined from a standard curve constructed by plotting the absorbancy at 650 nm of a 1.0 ml volume of culture (in a cuvette with a light path of 1.0 cm) vs. the viable cell counts. Viable counts were determined by plating an appropriate dilution of the culture and incubating overnight at 37°.

C. Preparation and Purification of Phage R17

1. Cell Lysis and the Partial Purification of the Released Phage by a Two-Phase Polymer System

Ten liter cultures of E. coli WP156 were grown in supplemented TMM medium at 37° in a small fermenter at a stirrer speed of 120 rpm. Under these conditions, cultures generally reached a cell density of 4×10^8 cells/ml about 6 to 6 1/2 hrs after inoculation and were then infected by the addition of a phage R17 suspension at a multiplicity of 5 - 10 PFU/cell. The stirrer speed was reduced to 50 rpm for 15 min after infection and was then increased to 140 rpm. After about 6 hrs, cell lysis was discernible through the appearance of clumps of cell wall debris accompanied by a reduction in the optical density of the infected culture. Cell lysis was completed by adding lysozyme (66 mg/l) and incubating 30 min and finally by adding chloroform (3 ml/l) followed by a further incubation for 20 min. The titer of crude lysate thus obtained varied from 5×10^{11} to 2×10^{12} PFU/ml.

The phage in the crude lysate was concentrated and partially purified by using a modification of the liquid two-phase polymer method described by Albertsson (1967). Following addition of 2.1 g sodium dextran sulfate (NaDS), 71.5 g polyethylene glycol 6000 (PEG) and 18 g NaCl per liter of crude lysate, the mixture was stirred to homogeneity prior to storage at 4° for at least 48 hrs to allow phase formation. Then, most of the top PEG phase (comprising 90 - 95% of the total volume) was siphoned off, care being taken not to disturb the interphase. The lower NaDS phase and the interphase material

containing cell debris and the highly concentrated phage were mixed with 50 ml SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and centrifuged at 10,000 g for 10 min. The supernatant was retained and the pellet washed four more times with 50 ml SSC. To the combined washings was added 0.15 vol of saturated KCl solution and this was stood for 2 hrs at 4° to precipitate the residual NaDS. After centrifugation at 10,000 g for 10 min, the supernatant was dialyzed for 24 hrs against two changes of SSC at 4°. The phage was pelleted by centrifugation at 18,000 rpm for 18 hrs in the Beckman Type 19 rotor and finally resuspended in 60 ml SSC in preparation for banding in a CsCl density gradient.

2. CsCl Isopycnic Banding of Phage

Preformed CsCl gradients were prepared from a conventional gradient maker by using equal weights rather than equal volumes of CsCl solutions at densities of 1.2 g/cc and 1.5 g/cc. The total volume of the gradient was 20 ml in a 35 ml cellulose nitrate gradient tube. Fifteen ml amounts of the R17 suspension were layered on top of the gradients and centrifugation was carried out at 23,000 rpm for 19 hrs at 4° using the SW 27 rotor of a Beckman L2-65B ultracentrifuge. Under these conditions, the phage particles form a band about three-quarters of the way down the tube. The phage is recovered by inserting a needle just below the band and collecting in a syringe and then the phage suspension is dialyzed against SSC at 4° overnight to remove the CsCl.

3. Assay for Phage Density

The number of phage particles in a phage suspension was determined from optical density measurements with a Beckman (DBG) spectro-

photometer, using an extinction coefficient at 260 nm of 7.66/mg/ml/cm (Gesteland and Boedtker, 1964). Assuming a molecular mass of 3.6×10^6 daltons per phage particle, as reported by the foregoing workers, one absorbancy unit was calculated to contain 2.18×10^{13} particles. A yield of about 30 ml of phage at a density of $1 - 5 \times 10^{15}$ particles/ml was usually obtained from a ten liter crude lysate.

D. Radioactive Labeling of Phage R17

1. ^{32}P- Labeling of R17 Phage RNA

Overnight cultures of E. coli WP156 were grown in supplemented TMM medium with the phosphate concentration lowered to 0.5 mM. A two ml aliquot of the overnight culture was transferred to 100 ml of fresh medium with a similar phosphate concentration and the cells grown up to 4×10^8 cells/ml. Phage infection was carried out at a multiplicity of 40 PFU/cell. At 5 min post infection, a neutralized solution of ^{32}P inorganic phosphate (1.0 mCi) was added and incubation continued. Purified phage obtained from such crude lysates normally had a specific radioactivity of 2×10^{-7} cpm/particle.

2. Labeling of R17 Phage A Protein with Radioactive Histidine

The fate of the A protein during phage infection can be followed by labeling the phage with radioactive histidine. This procedure exploits the fact that the A protein is the only histidine-containing polypeptide associated with wild-type phage (Steitz, 1968a). Labeling of the phage was carried out using ^3H-histidine according to the method originally developed by Krahn (1971). A 100 ml culture of E. coli WP156 was grown to a density of 4×10^8 bacteria/ml in supplemented TMM medium

to which 10 µg/ml of amino acids (no histidine), and 10 µg/ml each of urocanic acid, adenosine, guanosine, cytidine and uridine were added at the time of infection. The multiplicity of infection was 40 PFU/cell. Fifteen min after infection, 2,5-^{3}H-histidine was added to a final activity of 37.5 µCi/ml, and 7 min later, the pulse was terminated by the addition of unlabeled histidine to a final concentration of 10 µg/ml. Incubation of the culture was then continued until lysis occurred 3 - 4 hrs later. Purified phage obtained from such crude lysates generally had a specific radioactivity of 1×10^{-8} cpm/particle, and contained about 70 - 85% of the radioactivity in the A protein. The remainder of the radioactivity was found to be in the phage RNA, equally distributed between adenine and guanine residues (Krahn, 1971).

3. Purification of Radioactively Labeled Phage

Purification of the radioactively labeled phage was carried out using a slightly modified version of the procedure for unlabeled phage. After addition of PEG, NaDS and NaCl to the crude lysate, the mixture was stood for 18 hrs to ensure phase formation. Then it was centrifuged at 10,000 g for 10 min prior to removal of the PEG layer. The interphase material was retained and the pellet was washed 5 times with 5 ml amounts of SSC. After removal of the residual NaDS by KCl precipitation, the phage were pelleted by centrifugation for 2 ½ hrs at 4° in the Beckman 60 Ti rotor and were finally resuspended in 4 ml of SSC to which was added 2.4 g CsCl. Density gradient centrifugation was carried out at 35,000 rpm for 18 hrs at 4° using the Beckman SW 50.1 rotor and fractions were collected by piercing the bottom of the tube with a hollow needle.

E. Hot and Cold TCA Radioassay

Samples (30 - 100 µl)to be assayed for TCA-insoluble material were transferred by pipette to 3MM Whatman filter discs (2.1 cm diameter) pinned to a board. The paper discs were processed by a scheme modified from that published by Mans and Novelli (1960). For reasons of safety, ethanol was used in place of ether.

1. Hot TCA-Insoluble Products

Table 2.1

Preparation of Paper Discs for Hot TCA Insoluble Products

Step	Time	Wash medium	Temperature
1	30 min	10% TCA	4°
2	5 min	5% TCA	4°
3	45 min	5% TCA	90°
4	15 min	5% TCA	4°
5	15 min	85% EtOH	20°
6	15 min	90% EtOH	20°

2. Cold TCA-Insoluble Products

Treatment was similar to that shown in the above scheme with the substitution of a 15 min 5% TCA (4°) wash for step 3.

F. Radioisotope Counting

Dry samples such as radioactive material embedded in air-dried filter discs after TCA treatment were counted in 5.0 ml toluene-based scintillation fluor (prepared by the addition of 4 g Omni-fluor from New England Nuclear per liter of scintillation grade toluene) in a Beckman LS-230 liquid scintillation spectrometer.

Aqueous samples were assayed by combining 0.5 - 1.0 ml samples with 10 ml Scinti Verse (Fisher Scientific Co.) in a scintillation vial and shaking the mixture to homogeneity. The efficiency of counting in Scinti Verse was >95% for ^{32}P and >30% for ^{3}H.

External standardization was used to monitor the efficiency of counting of all radioactive samples.

Samples with low levels of radioactivity were counted for a sufficient length of time to reduce the error to less than 5%. All sample values were corrected for background radioactivity registered in controls containing no added sample.

G. Plaque Assay for Infectious Phage

The number of infectious phage in a suspension was determined by serially diluting the sample and mixing 1.0 ml of a dilution with 0.2 ml of seed culture (4×10^8 cells/ml) and 2.0 ml of liquefied top agar. After agitation, the mixture was poured onto hard agar in a Petri dish and allowed to solidify before the plates were inverted and incubated at 37° overnight before scoring for plaques. A single dilution was usually plated out in duplicate.

H. Isolation of RNA

RNA was isolated from R17 particles according to the procedure developed by Sagik et al., (1962). The phage particles were suspended to a concentration of 3 mg/ml in Sagik's buffer (0.01 M Tris HCl, pH 7.3, 0.01 M KCl, 0.005 M $MgCl_2$) containing 1% SDS and shaken for 5 min at 20°. Deproteinization was accomplished by adding an equal volume of buffer-saturated phenol containing 0.2% Macaloid and 0.1% SDS and shaken for 15 min at 4°. The phenol was redistilled immediately prior to use. The resulting emulsion was separated into phases by centrifugation at 10,000 g for 10 min and the aqueous layer was removed. The phenol extraction procedure was repeated three times. The RNA was then precipitated by the addition of two vol of cold (-20°) MeOH and 0.01 vol of 20% potassium acetate, pH 5.2 and was stored overnight at -20°. The precipitated RNA was then collected by centrifugation, dried under nitrogen gas and stored in a dessicator at -20° until used.

I. Sucrose Gradient Techniques

Linear (5 - 20%), 4.6 ml sucrose gradients in the appropriate buffer were poured from a two-chamber Buchler gradient maker. Before using, the gradients were equilibrated by storing at 4° for 6 hrs. Sucrose gradients incorporating 8 M urea or guanidine-HCl at the appropriate concentration were similarly prepared. In experiments where naked RNA was present, RNase-free sucrose was used in the preparation of the gradients.

Aqueous samples (0.1 - 0.3 ml) were layered carefully onto the

gradients without disturbing the meniscus. Centrifugation was carried out at an appropriate speed and temperature using the SW 50.1 rotor in the Beckman L2-65B ultracentrifuge.

Fractions from gradients were collected by piercing the bottom of the tube with a hollow needle.

J. Amicon Ultrafiltration

After lactoperoxidase treatment, iodinated phage particles were concentrated by ultrafiltration through an Amicon Minicon-S125 (exclusion limit = 125,000 daltons) which was prerinsed with bovine serum albumin (1 mg/ml) prior to use.

K. Techniques Used in the Analysis of Proteins by Gel Electrophoresis

1. SDS-Polyacrylamide Gel Electrophoresis

Disrupted phage proteins were analyzed by the SDS-polyacrylamide system described by Weber and Osborn (1969). Three different acrylamide concentrations were used:

(i) 7.5% acrylamide (w/v) - 0.2% N,N'bis-methylene acrylamide (w/v). These gels of dimensions 5 cm x 1 cm, were used in the preparation of purified A protein.

(ii) 10% acrylamide (w/v) - 0.28% N,N'bis-methylene acrylamide (w/v). Gels of this composition and with dimensions 10 cm x 6 mm were used in the analysis of lactoperoxidase iodinated R17.

(iii) 10% acrylamide (w/v) - 0.14% N,N'bis-methylene acrylamide (w/v). These gels, also of dimensions 10 cm x 6 mm were used in the analysis

of phage proteins which had been cross-linked with bifunctional reagents.

The gels which contained 0.1% SDS and 0.01 M sodium phosphate, pH 7.2, were polymerized chemically by the addition of 0.07% ammonium persulfate (w/v) and 0.04% TEMED (w/v). After addition of the latter, the gel solution was passed through a Gelman Metricel GA-4 membrane filter (pore size, 0.8 μ) and was de-aerated under vacuum. The gel tubes which were 1.0 cm longer than the required gel length were treated with Column Coat before use. After pouring each gel, a few drops of water were carefully layered on top. The protein samples were suspended in a solution with the following composition: 0.02 M sodium phosphate, pH 7.2, 1% SDS, 8% glycerol, 0.002% bromophenol blue and 5% 2-mercaptoethanol. The latter was omitted from samples containing proteins which had been cross-linked with methyl 4-mercaptobutyrimidate. The proteins were completely dissociated by immersing the samples for 5 - 7 min in boiling water. After layering the cooled samples (0.1 - 0.2 ml) on top of the gels, electrophoresis was carried out in a phosphate buffer (0.01 M sodium phosphate, pH 7.2, 0.1% SDS) at 5 - 8 mA/gel until the dye marker reached the bottom of the gel.

2. Staining of Gels

Gels were stained with Coomassie Blue by immersing in a solution containing 10% acetic acid, 25% isopropanol and 0.005% Coomassie Blue, overnight. Next day, the gels were transferred to a solution containing 10% acetic acid, 10% isopropanol and 0.005% Coomassie Blue. The latter solution was changed after 8 hrs and the following day, the gels were destained by immersing in 10% acetic acid.

3. Counting Radioactivity in Gels

The gels were fitted into a teflon block with calibrated (1.67 mm) transverse slits and were fractionated using a multiwire slicing device. Each slice was inserted into a scintillation vial to which was added 1.0 ml of a mixture of concentrated NH_4OH and NCS solubilizer in a 20:1 (v/v) ratio. The vials were then capped and incubated at 40° for 12 hrs. Then, the caps were removed and the contents dried in a 60° oven. After cooling, 5 ml of a toluene-based fluor was added to each vial and after standing in the dark at room temperature for 8 hrs (after which time the chemiluminescence has subsided), the radioactivity in each sample was monitored

L. Preparation of an A Protein-RNA Complex and its Analysis by Density Gradient Centrifugation in Cs_2SO_4

1. Preparation of an A Protein-RNA Complex

Acetic acid pellets of the phage were prepared as described (Osborn et al., 1970) by adding 2 ml of chilled glacial acetic acid to 1 ml (containing 1 mg) of a cold suspension of a mixture of ^{32}P-labeled phage (1.9×10^{-9} cpm ^{32}P/particle) and ^{3}H histidine-labeled phage (5×10^{-10} cpm ^{3}H/particle). The mixture was shaken gently at 4° for 1 hr and the precipitate harvested by centrifugation (10,000 g, 10 min). The pellet was washed first at 4° with 3 ml of 66% acetic acid to remove residual coat and then with 1 ml of buffer (0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA) to remove excess acid. The pellet was dried gently under a stream of nitrogen gas, dissolved in 2 ml of buffer (0.1 M Tris-HCl, pH 7.5, 0.001 M EDTA) and then added to 3 ml of a

saturated Cs_2SO_4 solution (made up in the same buffer) in preparation for density gradient centrifugation.

2. Stability of the A Protein-RNA Complex in Ethylene Glycol and Dimethyl Sulfoxide (DMSO)

The stability of the complex obtained by acetic acid treatment of intact particles as described above, was tested in 5% and 10% ethylene glycol and in 5% and 10% DMSO. The acetic acid pellet was dissolved in 2 ml of buffer (0.1 M Tris-HCl, pH 7.5, 0.001 M EDTA) containing the reagent and was incubated for 30 min at 4° prior to addition of 3 ml of a saturated Cs_2SO_4 solution.

3. Density Gradient Centrifugation in Cs_2SO_4

Centrifugation was carried out for 65 hrs at 4° and 35,000 rpm in the SW 50.1 rotor using the L2-65B ultracentrifuge. Thirty-six fractions were obtained by piercing the bottom of the tube and collecting 12-drop aliquots in a series of 13 mm tubes. In order to compensate for changes in surface tension, 14-drop fractions were collected from gradients containing DMSO. The average density of the fractions was determined refractometrically. The density profiles of gradients containing ethylene glycol or DMSO could not be obtained refractometrically since each of these solvents has a high refractive index of its own. The RNA concentration of each fraction was estimated from the ^{32}P counts precipitated by 5% TCA at 4° while the concentration of A protein was estimated from the ^{3}H counts precipitated by 5% TCA at 90°.

4. Nirenberg Assay

To determine whether or not the RNA in the fractions from the Cs_2SO_4 gradient (see above) contained bound protein, the Nirenberg

filtration assay was employed (Nirenberg and Leder, 1964). An aliquot of each fraction was diluted with 3 ml of cold buffer (0.1 M Tris-acetate, pH 7.2, 0.02 M magnesium acetate, 0.05 M KCl) and this was passed through a membrane filter (Millipore Filter Corp., type HA, pore size 0.45 μ). The filter was then washed three times with 5 ml amounts of cold buffer. Unbound RNA is removed in the wash while protein-bound RNA is retained on the filter, which is then dried and counted in 5 ml of toluene-based scintillation fluor, as described above.

M. Treatment of R17 with Guanidine-HCl (G-HCl) or Urea

The desired concentration of guanidine-HCl was obtained by dialysis against 6 M G-HCl in TKC buffer (0.04 M Tris-HCl, pH 7.3, 0.1 M KCl, 0.005 M $CaCl_2$) at 4°. The concentration of reagent was monitored refractometrically. Treatment of the phage with 8 M urea was accomplished by the addition of 0.8 vol of 10 M urea in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.3) to 0.2 vol of phage suspension.

N. Neutralization of Phage Infectivity with Anti-(A Protein)-Antibodies

1. Isolation of Pure A Protein and Preparation of Antisera

The A protein was prepared from intact R17 particles using the acetic acid-dodecyl sulfate procedure developed by Osborn et al., (1970). One vol of a phage suspension (1 - 4 mg/ml in SSC at 4°) was added to 2 vol glacial acetic acid which had been cooled in an ice bath with stirring until the acid had just begun to freeze. The mixture was shaken for 1 hr at 4° and then the precipitate was removed by centrifugation (10,000 g, 10 min). The supernatant containing the coat protein

was removed and the pellet was washed first at 4° with 66% acetic acid to remove residual coat protein and the at room temperature with 95% ethanol to remove the excess acid.

The washed pellet was then dissolved in sufficient 1% SDS to give an RNA concentration less than 10 mg/ml and the pH was adjusted to 7.0 by the addition of 1 N NaOH. The solution was the made 0.1 M in K^+ by addition of 2 M KCl, warmed to 50° to dissolve the potassium dodecylsulfate precipitate and then cooled to 4° until the precipitate formed again. This precipitate, which contained more than 50% of the A protein was harvested by centrifugation (10,000 g/10 min) and the A protein remaining in the supernatant was recovered by adding solid SDS to a final concentration of 1%, restoring the KCl concentration to 0.1 M and repeating the precipitation. Both precipitates were washed twice with 0.5 M KCl, and then transferred while warm to a sac for dialysis against 0.05 M $NaHCO_3$, pH 8.1, 0.1% SDS at 37° overnight. To free the A protein from dodecylsulfate, nine parts of acetone were added to one part of redissolved potassium dodecylsulfate pellet. The resulting A protein precipitate was harvested by centrifugation (10,000 g/10 min).

The A protein prepared in this manner is contaminated with a small amount of coat protein and RNA but it can be further purified by SDS-polyacrylamide gel electrophoresis. The precipitate remaining after acetone washing was dissolved in a phosphate buffer containing 0.1% SDS and this was stained as described by Griffith (1972) with a Remazol dye (Amylose Azure) during the denaturation prior to electrophoretic analysis. The migration of proteins stained in this manner can be visualised, thus permitting termination of electrophoresis

when optimum resolution has been obtained. A typical protocol for this procedure is as follows. The A protein precipitate was dissolved in 0.01 M sodium phosphate buffer, pH 7.2 containing 0.1% SDS, to a concentration of approximately 0.5 mg/ml. To 1 ml of this solution were added 50 µl 1.0 M Na_2HPO_4, pH 9.2 and 25 µl 1% Amylose Azure in 10% SDS. This mixture was boiled 15 min and then 2 ml of the unstained A protein preparation were added. Glycerol and 2-mercaptoethanol were added to give final concentrations of 5% and 4%, respectively. The mixture was boiled 3 min, cooled and 0.2 ml aliquots were subjected to SDS-polyacrylamide gel electrophoresis, as described. Electrophoresis was carried out at 8 mA/gel for 3 1/2 hrs at which time the A protein was clearly separated from the coat protein. To ensure that the unstained A protein had the same mobility as that stained by Amylose Azure, both preparations were subjected to electrophoresis separately and were then stained with Coomassie Blue. The mobility of the A protein was found to be identical in each case. Also, the mobility of ^3H-histidine labeled A protein was found to be similar to that of the Amylose Azure stained A protein.

The A protein bands were cut out of the gels and after addition of a small amount of saline, were macerated by repeated passage through a syringe. The A protein -acrylamide mixture was then injected subcutaneously in the dorsal region of New Zealand rabbits. Each injection comprised material from 5 gels (0.5 mg A protein). Four injections were administered over a 5 month period. The rabbits were bled 11 days after the final injection and the serum was stored at -20°.

For immunodiffusion analysis, the A protein was purified by cutting out the bands from gels as described above, emulsifying these

in water and applying to a second gel to the end of which a dialysis bag was attached. Highly purified A protein was recovered from the dialysis bag.

2. Micro-immunodiffusion Analysis

Double diffusion analysis was carried out on standard microscope slides using 1% agarose dissolved in 0.1 M sodium phosphate, pH 7.2, 0.15 M NaCl, 0.1% SDS. This was prepared by boiling 20 min, filtering (Gelman Metricel GA-4, pore size 0.8 μ) and making up to the original volume. The SDS was included to solubilize the A protein. A plexiglass template, in which six wells are situated circularly around a central well, was seated on four pieces of 20 lb fishing line (one piece at each corner of the slide) to allow preparation of the gel. The agarose solution was maintained at 50° while pouring and after gelation had occurred (by standing at room temperature for 10 min), the wells were cleared of agarose by means of a vacuum line.

After application of antigen and antibody, the slides were incubated in a water-saturated atmosphere at room temperature for 72 hrs. Then the templates were removed and the slides immersed for 48 hrs in a shaking bath containing phosphate-buffered saline (0.01 M sodium phosphate, pH 7.2, 0.9% NaCl) at 4° in order to remove miscellaneous proteins. After washing by immersing for 3 - 4 hrs in distilled water at 4°, the slides were stained for 1 hr in Coomassie Blue (7.5% acetic acid, 5% methanol, 0.05% Coomassie Blue — filtered through a Gelman Metricel GA-4 membrane prior to use). Destaining was accomplished in 7.5% acetic acid.

3. Neutralization Assay

The phage neutralization was essentially that described by

Adams (1959). Approximately 1 x 10^6 PFU of phage in 0.1 ml were added to 4.9 ml of a dilution of antiserum and the mixture was incubated at 37°. At zero time and at appropriate time intervals thereafter, 0.1 ml samples were removed and diluted with saline at 4° to prevent further phage inactivation. Aliquots of these dilutions were plated out as described above.

The neutralization constant, K, was calculated from the following relationship:

$$K = \frac{2.3\ D}{t} \log_{10} (P_o/P)$$

in which D is the dilution of serum, t is the incubation time in minutes, P_o is the plaque assay at zero time and P is the plaque assay at time t.

4. Purification of Immunoglobulin from Anti-(A Protein)serum

The purification of 7S gamma globulin (IgG) from anti-(A protein)serum was carried out as follows. To 5 ml antiserum was added 2.5 ml saturated $(NH_4)_2SO_4$. The resulting precipitate was harvested by centrifugation (10,000 g, 10 min) at 4° and then resuspended in 5 ml phosphate-buffered saline (0.01 M sodium phosphate, pH 7.5, 0.9% NaCl). The $(NH_4)_2SO_4$ precipitation was repeated twice and the IgG precipitate was resuspended in 0.01 M sodium phosphate, pH 7.5, and then dialyzed overnight at 4° against 2 changes of 0.01 M sodium phosphate, pH 7.5, (700 ml buffer/ml of resuspended IgG) in preparation for purification by DEAE cellulose using the batchwise technique described in the Whatman laboratory manual, "Advanced Ion-exchange Celluloses".

To 35 g of DEAE cellulose (Whatman DE 52) — which was fully

equilibrated with 0.01 M sodium phosphate, pH 7.5, cooled to 4° and drained free of excess buffer — was added the dialyzed IgG and the mixture was stirred thoroughly and then stood for 2 hrs at 4° with occasional stirring. Then, 20 ml 0.01 M sodium phosphate, pH 7.5, was added and after stirring, the liquid phase was collected by centrifugation at 10,000 g for 10 min. This liquid phase was then filtered through a Gelman Metricel membrane filter (GA-4, pore size 0.8 μ) and concentrated by ultrafiltration (Amicon XM100A, exclusion limit 100,000 daltons).

The IgG purified in this manner contained no contaminating polypeptides, as judged by SDS-polyacrylamide gel electrophoresis.

O. Lactoperoxidase Catalyzed Iodination

To 750 μg R17 (purified as described above except that the final step in purification was sedimentation in a sucrose gradient rather than equilibrium density gradient centrifugation in CsCl) in SSC were added 5 μg lactoperoxidase, 0.5 μg NaI and 0.1 mCi of $Na^{125}I$. The reaction was initiated at room temperature by the addition of 10 nmoles hydrogen peroxide which gave a final concentration of 8.4×10^{-5} M. Three further 10 nmole aliquots of hydrogen peroxide were added at 1 min intervals. Six min after initiation, the reaction was terminated by dilution with buffer. Unreacted lactoperoxidase and iodine were removed by ultrafiltration, as described above, followed by overnight dialysis against SSC at 4°.

P. Cross-linking Studies

Intact R17 particles were treated with the following bifunctional cross-linking reagents:

(a) Methyl 4-Mercaptobutyrimidate (MMB)

Cross-linking with MMB was carried out using the method described by Sun et al., (1974). Intact phage particles at a concentration of 3.5 mg/ml were incubated with the appropriate concentration of MMB in 0.05 M triethanolamine-HCl, pH 8, 0.001 M $MgCl_2$, 0.05 M KCl and 3% 2-mercaptoethanol for 30 min at the appropriate temperature. These conditions led to the addition of sulfhydryl-containing chains to the reactive amino groups on the phage proteins. After overnight dialysis at 4° against the same buffer minus 2-mercaptoethanol, the sulfhydryls were oxidised by adding hydrogen peroxide to a final concentration of 40 mM and incubating at room temperature for 30 min. This reaction was readily reversed in 3% 2-mercaptoethanol.

(b) Dimethyl Suberimidate (DMS)

Cross-linking with DMS was carried out using the conditions outlined by Barritault et al., (1975). The appropriate amount of DMS was dissolved immediately before use in a mixture of Buffer A (0.1 M triethanolamine-HCl, pH 7.2, 0.01 M $MgCl_2$) and 2 N KOH (85:15 v/v). The pH of a suspension of phage particles (final concentration = 5 mg/ml) in Buffer B (0.1 M triethanolamine-HCl, pH 7.2, 0.01 M $MgCl_2$, 0.05 M KCl, 0.006 M 2-mercaptoethanol) was adjusted to pH 8.2 by the addition of 0.06 vol of buffer C (2 M triethanolamine-base, 0.05 M KCl, 0.01 M $MgCl_2$). To this was added 0.1 vol of the DMS solution and the mixture was incubated at 30° for 90 min.

(c) Glutaraldehyde

The reaction conditions used by Steck (1972) were employed for this study. A phage suspension (1 mg/ml) in 5 mM sodium phosphate, pH 8, was treated with 15 mM glutaraldehyde for 2 hrs prior to electrophoresis.

CHAPTER III

LOCATION OF THE A PROTEIN IN BACTERIOPHAGE R17

A. Introduction

It has been shown by Curtiss and Krueger (1974) that the A protein isolated from MS2 particles which have been conjugated with dinitrophenol (DNP) contains covalently attached DNP. This led them to suggest that the A protein is located on the surface of the virion. This suggestion was supported by their finding that lactoperoxidase catalyzes the iodination of the A protein as well as the coat protein monomers in intact MS2 particles. However, the location of the A protein is not conclusively demonstrated by these means. Dinitrophenyl-amino groups are not protonated at neutral or even acidic pHs and therefore, proteins so modified have altered electrostatic properties. Although Curtiss and Krueger (1974) showed that neither the density, the rate of sedimentation nor the infectivity is altered upon DNP treatment, it could be argued that such conjugation may alter the conformation of the A protein in such a way that it assumes a surface location. Also, iodination by lactoperoxidase cannot be absolutely relied upon as a means of establishing the surface location of a protein. Indeed, the procedure employed by Curtiss and Krueger for the purification of the MS2 particles included steps (freon extractions and CsCl density gradient centrifugation) which have been shown to permit iodination of a core polypeptide in adenovirus (Everitt et al., 1975).

The present report describes further investigations on the location of the A protein in RNA phage. It is shown that antibodies prepared against highly purified A protein neutralize the

the infectivity of the phage, and that lactoperoxidase iodination of intact R17 particles purified without using freon or CsCl centrifugation, results in the iodination of both the A protein and the coat protein. These findings, considered in conjunction with those obtained by Curtiss and Krueger (1974), overwhelmingly imply a surface location for the A protein of the RNA phages.

As a possible means of investigating the topography of the A protein on the virion, bifunctional cross-linking reagents were employed. The latter have been used to ascertain the neighbourhood relationship of protein components in ribosomes (Bickle et al., 1972; Chang et al., 1972; Slobin, 1972; Clegg and Hayes, 1974) and in the plasma membrane (Ji and Ji, 1974). The reagents used in the present study are methyl 4-mercaptobutyrimidate (MMB), dimethyl suberimidate (DMS) and glutaraldehyde. No information regarding the location of the A protein relative to the coat protein monomers could be gleaned from these cross-linking studies but the formation of coat protein dimers was noted and this will be discussed in relation to prevalent theories regarding the structure of the RNA phages.

B. Results

1. Immunodiffusion

Fig. 3.1 illustrates the reaction of anti-(A protein)serum with A protein. It may be seen that A protein-antibody precipitin bands were produced at the two highest concentrations of antiserum used. That an immunoprecipitate might be detectable in the presence of 0.1% SDS was suggested by the findings of Crumpton and Parkhouse (1972) who showed variable degrees of inhibition of precipitation by 0.1% SDS.

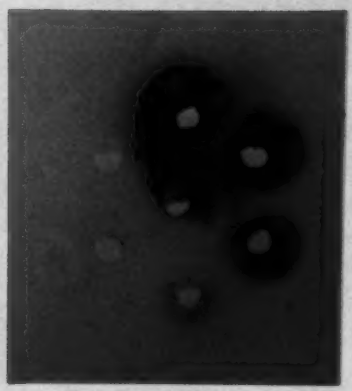


Fig. 3.1 Gel diffusion pattern of A protein and anti-(A protein) serum. The central well contains 5 µl of A protein dissolved in 0.01 M sodium phosphate, pH 7.2, 0.1% SDS at a concentration of 1 mg/ml. Contents of outer wells beginning in the topmost well and going clockwise: 5 µl of the following serial dilutions of antiserum (undiluted, 1/2, 1/4, 1/8, 1/16) and 5 µl of phosphate-buffered saline.

No precipitin line was observed when the A protein was replaced by the coat protein under similar conditions of immunodiffusion.

2. Neutralization of Phage Infectivity

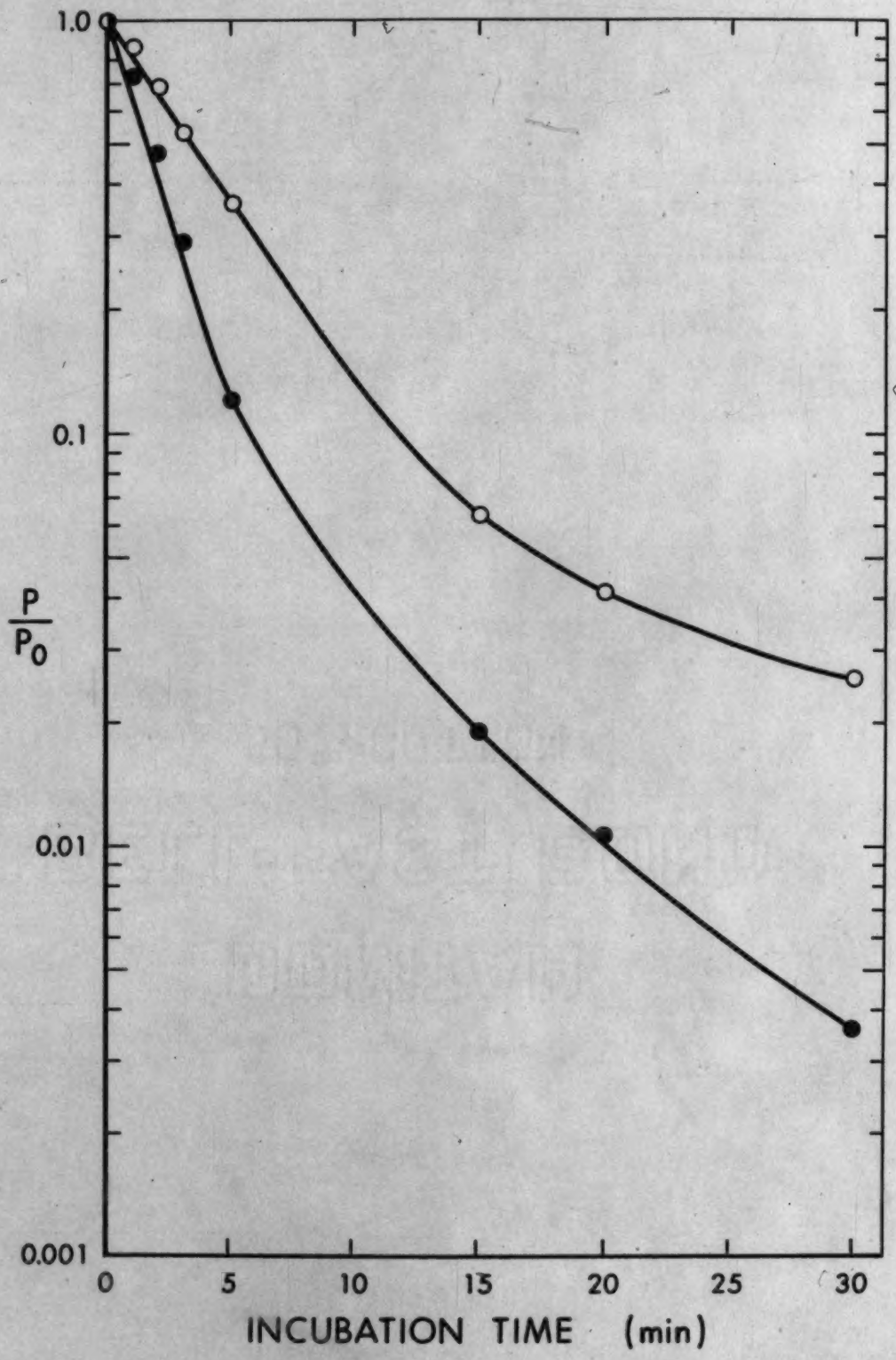
The kinetics of neutralization of bacteriophage R17 by antiserum to A protein were measured at antiserum dilutions of 1/100 and 1/200 and the results are shown in Fig. 3.2 as a plot of the logarithm of the fraction of surviving phage as a function of the incubation time of the reaction mixture. It can be seen that the rate of neutralization depends on the antibody concentration since the two-fold decrease in the latter increases the time for 90% neutralization from 5.8 min to 11.8 min. A similar neutralizing effect was observed when the phage were incubated with immunoglobulin purified from the anti-(A protein)serum by $(NH_4)_2SO_4$ precipitation and ion-exchange on DEAE cellulose, as described in Materials and Methods. In the control experiment in which the phage were treated with antiserum obtained from the rabbits prior to injection of the A protein, no decrease in infectivity was observed.

The neutralization constant, K, was found to be 43 min^{-1}. This relatively low value for K is not surprising in view of the highly denatured state of the A protein which was used as antigen. Also, it is probable that only a small part of the A protein is exposed on the virion; presumably, a large portion of the polypeptide is involved in interactions with the remainder of the particle.

3. Lactoperoxidase Catalyzed Iodination

By virtue of its high molecular weight (77,000), lactoperoxidase is unable to penetrate an intact cell membrane or cell wall. Therefore, in the case of a spherical capsid such as that possessed by the RNA phages, only those proteins which are on the surface of the capsid

Fig. 3.2 Neutralization of bacteriophage R17 by rabbit anti-(A protein)serum at 37°. The ordinate gives the logarithm of the ratio of plaques at a certain time to that at zero time. ●——● 1/100 dilution of antiserum, ○——○ 1/200 dilution of antiserum.


1.0
0.1
0.01
0.001
P/P0
0
5
10
15
20
25
30
INCUBATION TIME (min)

are expected to be iodinated by lactoperoxidase. The primary target of iodination in proteins is the phenolic group of tyrosine although the imidazole group of histidine may also be susceptible. This system has been used to identify the exposed proteins on ribosomes (Michalski et al., 1973), on erythrocytes (Morrison, 1974) and on viruses (Stanley and Haslam, 1971; Sefton et al., 1973).

In order to ascertain whether the enzymatic treatment had caused any disruption or distortion of the R17 particles, the iodinated phage were subjected to sucrose gradient centrifugation. The resulting sedimentation profile (not shown) was found to be identical to that of untreated phage indicating that the physical integrity of the particles remains unchanged following lactoperoxidase treatment.

When the iodinated phage were disrupted and analyzed by electrophoresis, two peaks were observed (Fig. 3.3) having mobilities corresponding to R17 coat protein and A protein. A small amount of material remained at the top of the gel and this is presumed to be a phage protein aggregate. No iodination occurred in the control experiments where either the H_2O_2 was omitted or where both the H_2O_2 and lactoperoxidase were omitted. It was found that the A protein represented approximately 9% of the total counts instead of just under 2% as would be expected from the molar ratio of tyrosine in the A protein [where there are 13 residues (Steitz, 1968b)] to that in the coat protein [where there are 4 residues (Weber, 1967)]. Even if all 5 histidines are iodinated in the A protein, this does not account for the discrepancy. It therefore appears that the A protein is more accessible to lactoperoxidase iodination than the coat protein monomers. Curtiss and Krueger (1974) found an even greater proportion of iodine in the A protein. The lower value obtained in the present study may be due to the less disruptive mode of purification employed.

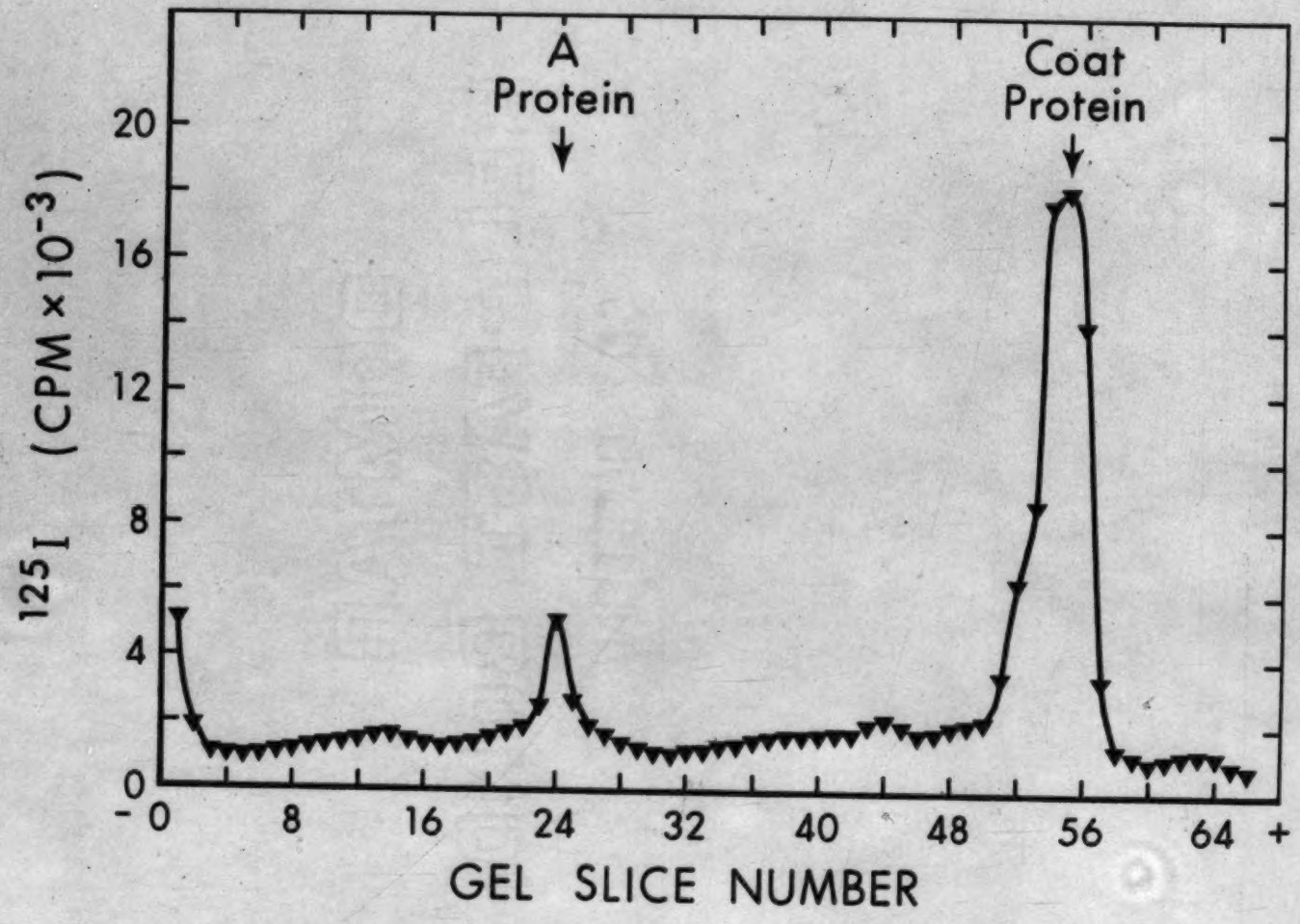


Fig. 3.3 SDS-polyacrylamide gel electrophoresis of lactoperoxidase ^{125}I-labeled R17. The iodinated phage particles were disrupted by boiling 3 min in 0.1% SDS and 40 µg of phage material was applied per gel.

4. Cross-linking

Each of the three reagents used in this study cross-link the amino groups of proteins. Both the A protein and the coat protein contain lysine residues [16 residues in A protein (Steitz, 1968b); 6 residues in coat protein (Weber, 1967)] and therefore should be capable of being cross-linked by these reagents. The extent of cross-linking of ^{3}H-histidine labeled phage was examined by SDS-polyacrylamide gel electrophoresis. The gels were stained in Coomassie Blue as described in Materials and Methods and photographs of these gels are shown in Figs. 3.4, 3.5 and 3.6. After staining, the gels were sliced and counted in toluene-based fluor, as described in Materials and Methods. The position of the ^{3}H peak is indicated on the figures.

Sucrose gradient analysis of the cross-linked phage particles revealed a similar sedimentation profile (not shown) to that of untreated phage. Hence, the physical integrity of the particles remains unchanged following treatment with the bifunctional reagents used in the present study.

Treatment of intact particles with MMB (Fig. 3.4) caused the formation of a major new band of which the electrophoretic mobility corresponds to that expected for coat protein dimers, (MW 28,000). Two additional complexes of molecular weights 50,000 to 54,000 and 76,000 to 80,000 are found. These are assumed to be tetramers and hexamers of coat protein monomers, respectively, rather than a complex containing A protein since upon cross-linking ^{3}H-histidine labeled R17 with MMB, all the ^{3}H counts coincided with the A protein band. Treatment with DMS (Fig. 3.5) similarly caused formation of dimers and also a small amount of material of molecular weight 52,000, again

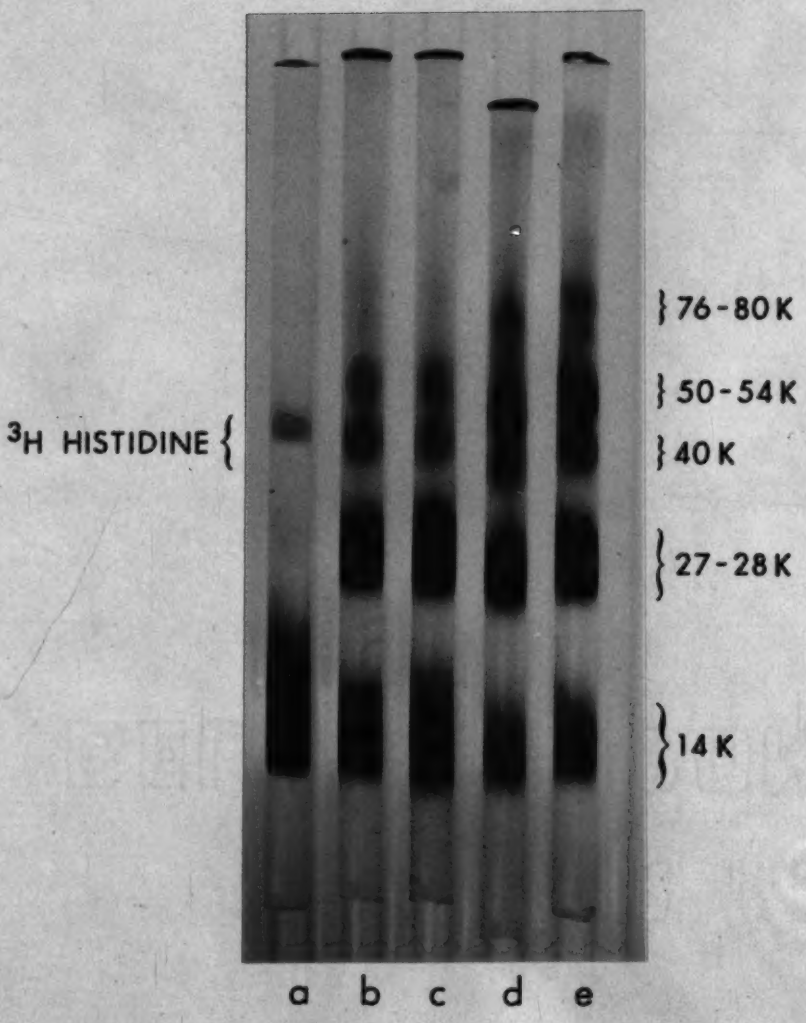


Fig. 3.4 Cross-linking of bacteriophage R17 with methyl 4-mercapto-butyrimidate (MMB). A suspension of R17 (3.5 mg/ml) was treated with (b) 10 mM MMB at 22° (c) 10 mM MMB at 37° (d) 20 mM MMB at 22° (e) 33 mM MMB at 22°, for 30 min and oxidised with H_2O_2 prior to electrophoretic analysis, as described in Materials and Methods. An aliquot of reaction mixture (b) was treated with 2-mercaptoethanol prior to electrophoresis and this is shown in (a).

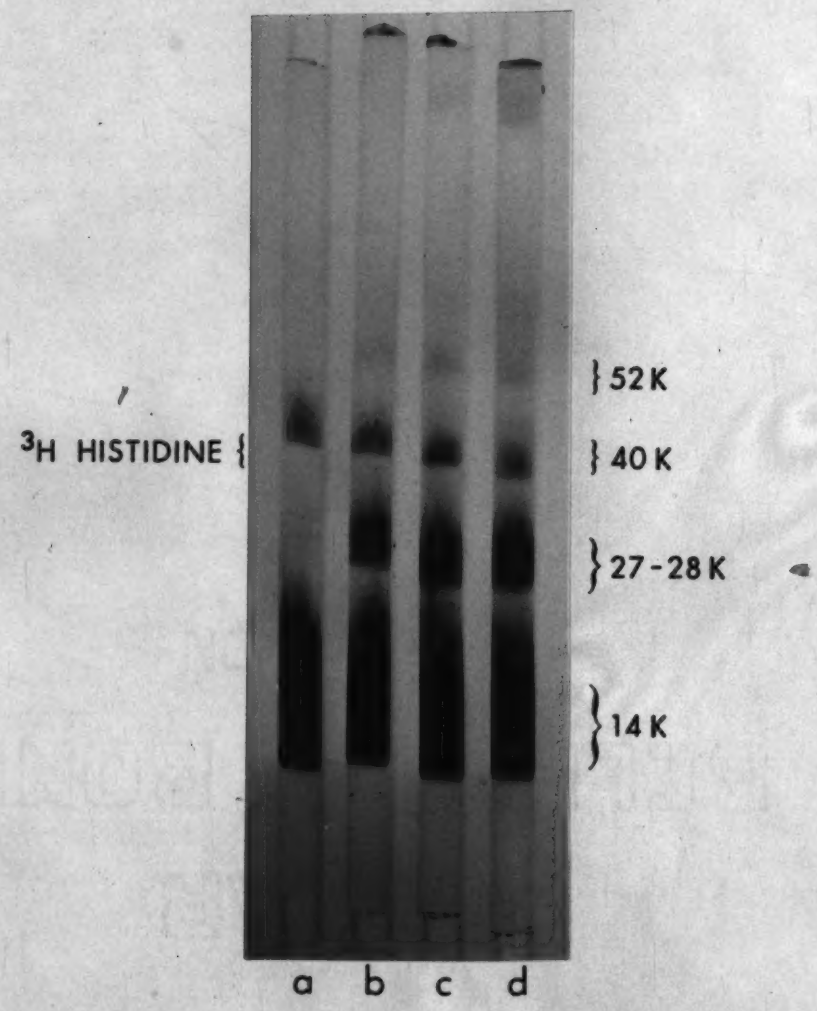


Fig. 3.5 Cross-linking of bacteriophage R17 with dimethyl suberimidate (DMS). A suspension of R17 (5 mg/ml) was treated with DMS for 2 hrs at 30° at the following concentrations (b) 8 mg/ml (c) 12.5 mg/ml (d) 17.5 mg/ml prior to electrophoretic analysis as described in Materials and Methods. An untreated sample of R17 is shown in (a).

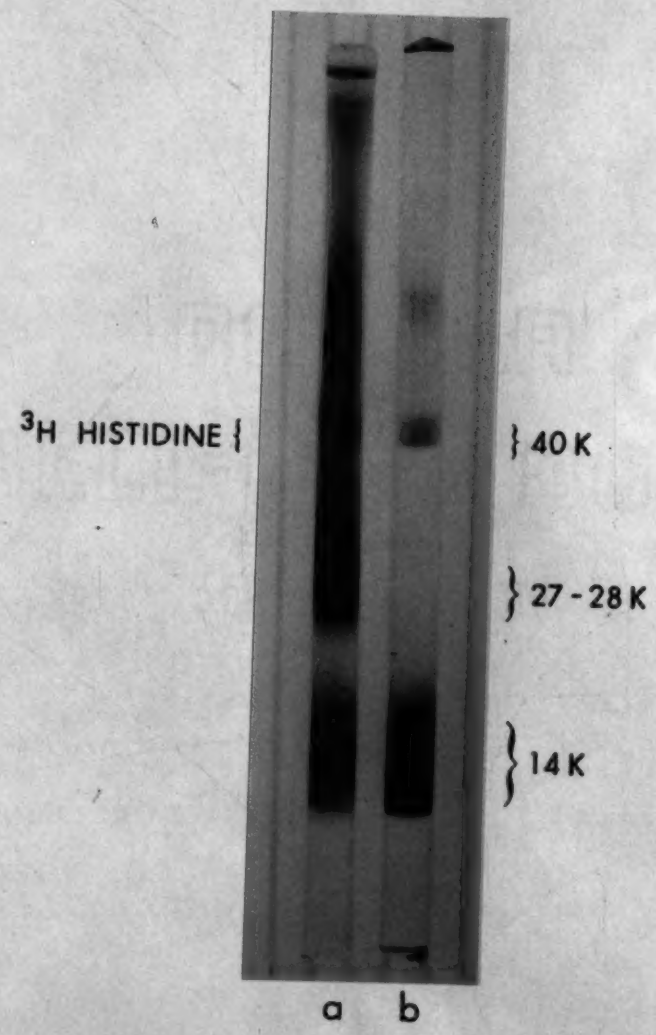


Fig. 3.6 Cross-linking of bacteriophage R17 with glutaraldehyde. (a) Phage R17 (1 mg/ml) was treated with 15 mM glutaraldehyde for 2 hrs at 22° prior to electrophoretic analysis as described in Materials and Methods. An untreated sample of R17 appears in (b).

presumably coat tetramers. The major species produced by glutaraldehyde (Fig. 3.6) was also dimers but in addition, this reagent caused the formation of a heterogeneous population of products of different molecular sizes. Of the concentrations tested, only the effect of 15 mM glutaraldehyde is shown. Lower concentrations of this reagent caused a proportionately lesser quantity of cross-linked product.

C. Discussion

Neutralization of the infectivity of phage R17 with antibody prepared against the A protein confirms the previous finding (Argetsinger and Gussin, 1966; Roberts and Steitz, 1967: Kaerner, 1970; Verbraeken and Fiers, 1972) that the A protein is essential for infectivity and provides direct evidence for the surface location of this protein in R17 particles. It has been previously observed that antibodies prepared against coat protein monomers have no effect on the infectivity of phage MS2 although capsid antiserum has a significant neutralizing effect (Rohrmann and Krueger, 1970). The capsids used in the preparation of the latter serum were generated by rapidly freezing and thawing phage particles and subsequently treating with RNase. Such capsids may contain residual A protein. However, even if A protein were removed by this treatment, it is easy to envision that adsorption of the antibodies to the region of the capsid in the vicinity of the A protein would sterically hinder the attachment of the latter to the F pilus.

The kinetics of neutralization were determined at antiserum dilutions of 1/100 and 1/200 and were found to be first order during the initial reaction period. After 5 min, the rate of inactivation

became slower, suggesting that the surviving phage are somewhat more resistant to the effect of antibody. Similar neutralization curves have been obtained for most of the phages studied and several theories have been proposed in an effort to account for this phenomenon (Adams, 1959; Krummel and Uhr, 1969). The mode of neutralization observed in the present study corroborates the previous finding that neutralization of f2 phage is due to a single "hit" by an antibody at some critical site on the surface of the virion (Witte and Slobin, 1972). Indeed, these authors suggest that this critical site could possibly be the A protein.

The ease of formation of coat protein dimers in the present study suggests an important role for this species in the structure of the phage. The maximum distances spanned by DMS and MMB are 11.7 Å and 14.6 Å, respectively, while the glutaraldehyde which is largely polymeric, causes heterogeneous cross-linking (Richards and Knowles, 1968). No cross-linking was observed when intact particles were treated with either tartryl diazide which spans 6 Å or o-phenanthroline/$CuSO_4$ which stimulates the air oxidation of sulfhydryls, using the reaction conditions outlined by Lutter (1974) and Steck (1972), respectively. This implies that a certain minimum distance must be bridged to permit dimer formation.

The recent model of phage capsid structure proposed by Dunker and Paranchych (1975) in which the protein subunits are arranged in dimer formation with rings of five about the five-fold axis and rings of six about the three-fold axis further emphasizes the importance of dimers in the geometry of the capsid. This model has been substantiated by the three-dimensional image reconstructions from electron micrographs

of phage particles (Crowther et al., 1975) and the latter authors suggest that assembly of the RNA phages may take place via a dimer intermediate. The model does not specify the location of the A protein but it is proposed (Dunker and Paranchych, 1975) that the latter could protrude through one of the openings at either the three-fold or the five-fold axes. The absence of a high molecular weight product containing A protein in the present study probably reflects the unavailability of reactive amino groups rather than the distance of the A protein from the coat protein monomers.

CHAPTER IV

THE ISOLATION OF AN INFECTIOUS A PROTEIN-RNA COMPLEX FROM BACTERIOPHAGE R17

A. Introduction

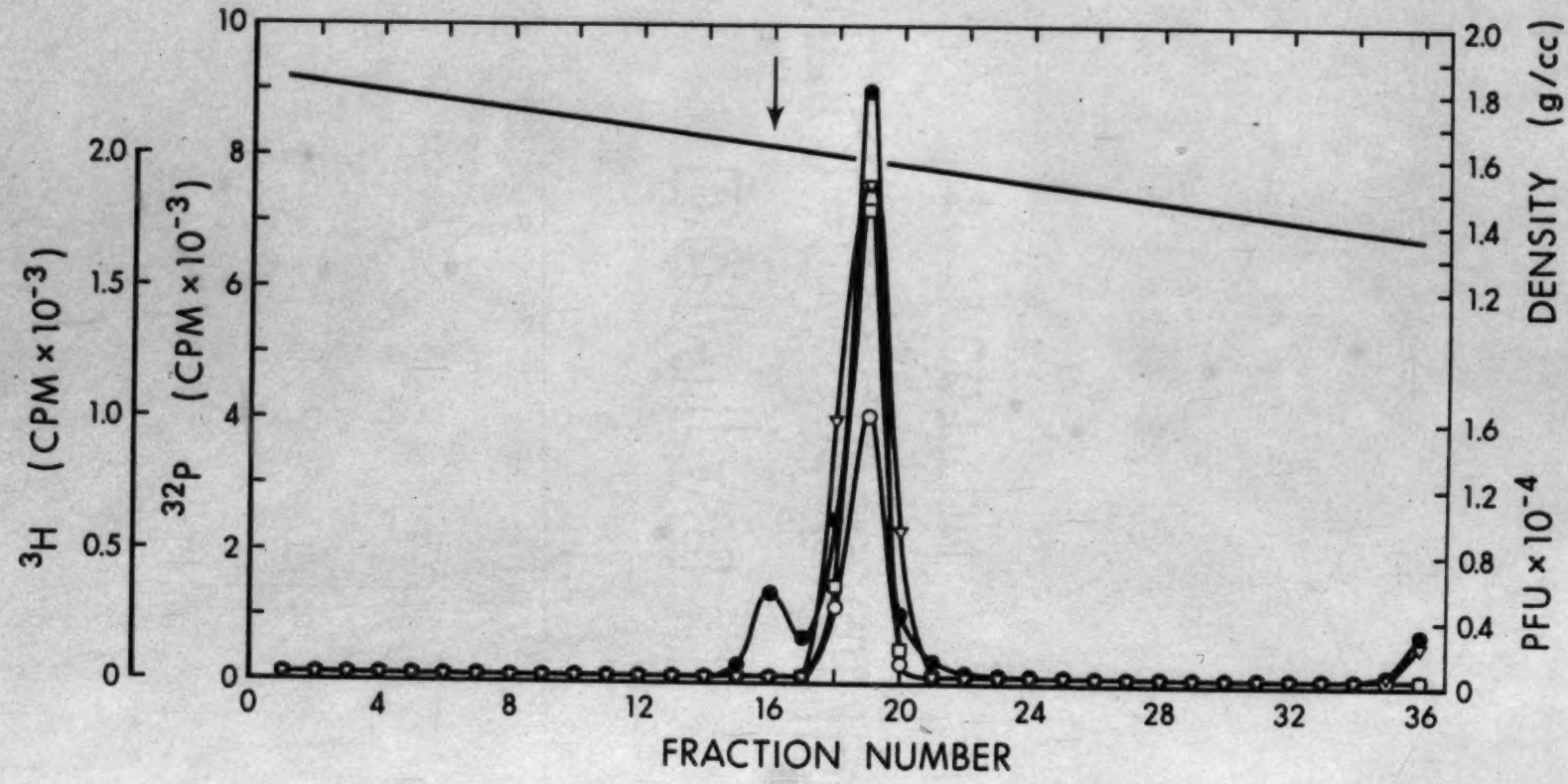
As previously discussed, Leipold and Hofschneider (1975) isolated an infectious A protein-RNA complex by sucrose gradient sedimentation of the acetic acid precipitate of bacteriophage M12. However, attempts in this laboratory to apply their isolation procedure to phage R17 failed because the A protein, rather than co-sedimenting with the RNA, was found to adsorb to the walls of the gradient tubes. Both cellulose nitrate tubes (capacity = 5 ml) and polyallomer tubes (capacity = 12 ml) were tried, but in each case, the A protein was almost totally removed from solution leaving free, partially-degraded RNA which sedimented as a single peak down the gradient. Pretreatment of the gradient tubes with Column Coat did not prevent the loss of A protein in this manner. However, as described in the present chapter, the problem was finally circumvented by using cesium sulfate centrifugation rather than sedimentation on a sucrose gradient as a means of isolating the complex.

B. Results

1. A Protein-RNA Complex on Cesium Sulfate Gradient

Intact phage particles were treated with 66% acetic acid as described in Materials and Methods and the resulting pellet was solubilized and then subjected to density gradient centrifugation in Cs_2SO_4 of mean density 1.6 g/cc. As seen in Fig. 4.1, two bands were present

Fig. 4.1 Cesium sulfate density gradient analysis of the solubilized acetic acid pellet prepared from a mixture of ^{3}H-histidine labeled and ^{32}P-labeled phage. The concentrations of RNA and A protein were estimated by subjecting 35 µl aliquots of each fraction to precipitation by TCA at 4° and 90°, respectively, as described in Materials and Methods. The remaining 70 µl of each fraction was diluted with 4.0 ml of Nirenberg buffer of which 1.0 ml was assayed for infectivity, while the remaining 3.0 ml were used to determine the amount of protein-bound RNA as described in Materials and Methods. The density profile which was obtained refractometrically is shown. The arrow indicates the position of phenol-extracted RNA in a parallel gradient. Centrifugation was from right to left. ●——● Cold TCA-insoluble ^{32}P cpm, ▽——▽ Hot TCA-insoluble ^{3}H cpm, □——□ Protein-bound RNA by Nirenberg assay, ○——○ Infectivity (PFU).


^{3}H (CPM × 10^{-3})
^{32}P (CPM × 10^{-3})
DENSITY (g/cc)
PFU × 10^{-4}
FRACTION NUMBER
0
4
8
12
16
20
24
28
32
36
0.5
1.0
1.5
2.0
2
6
10
0.4
0.8
1.2
1.4
1.6
1.8

at equilibrium. The band at higher density (1.63 g/cc) contains protein-free RNA (as judged by the Nirenberg assay), it is non-infectious and its position coincides with that of phenol-extracted phage RNA in the control gradient. The band at lower density (1.59 g/cc) contains RNA bound to A protein and this band contains the only infectious material in the gradient. This infectivity was totally abolished by incubation with 5 µg/ml pancreatic RNase at 37° for 30 min, under which conditions intact phage retain their total infectivity.

The molar ratio of protein-bound RNA to A protein in the complex was calculated from the known specific radioactivities of the RNA and A protein and found to be 1:1. However, this value varied from 0.4 to 1.0 mole of A protein per mole of RNA, depending on the amount of A protein that happened to adhere to the walls of the cellulose nitrate tube during centrifugation in the Cs_2SO_4 gradient and the amount of overlap between the peak containing RNA alone and the peak containing the A protein-RNA complex. That the counts on the tube were not simply due to precipitation was determined by cutting the tube into sections and counting each section in Scinti Verse. It was found that there was an approximately equal distribution of counts on the surface of the tube. The percentage of the total radioactivity which was found to adhere to the tube varied from 25% to 60% for ^{3}H and from 10% to 25% for ^{32}P.

In order to minimise loss of A protein by adherence to glassware and dialysis tubing (Hohn and Hohn, 1970; Steitz, 1968b; Valentine et al., 1969), the fractions were not dialyzed prior to assaying for infectivity. Nevertheless, the level of infectivity observed, viz., $1 - 2.2 \times 10^{-9}$ PFU per original infectious unit, was one hundred-fold lower than that reported by Leipold and Hofschneider (1975) for M12.

The low yield obtained in the present study may be due to the effects of high salt on the complex.

2. Dissociation of the A Protein-RNA Complex in Ethylene Glycol

The stability of the A protein-RNA complex in ethylene glycol was investigated. This reagent has been shown to disrupt hydrophobic interactions in proteins (Sage and Singer, 1962) and in a synthetic polynucleotide (Fasman et al., 1964b). Hydrogen bonds are expected to become more stable in ethylene glycol due to its low dielectric constant and this effect is presumed to account for the increased helical content which is observed when polyglutamic is dissolved in this solvent (Fasman et al., 1964a). In the present study, ethylene glycol causes the dissociation of the complex of density 1.59 g/cc and induces the formation of a new low density complex containing a higher molar ratio of A protein to RNA. Fig. 4.2 A shows the profile obtained when the acetic acid pellet is dissolved in 5% ethylene glycol prior to centrifugation. Here, the original complex is entirely dissociated and a small amount of the new complex appears at a density of approximately 1.48 g/cc. (The latter value was determined from a parallel gradient; the actual density of the complex could not be obtained refractometrically since ethylene glycol itself has a high refractive index). When the acetic acid pellet is dissolved in 10% ethylene glycol (Fig. 4.2 B) prior to centrifugation, a similar profile is obtained except that the low density complex is present in about 3.5 times the quantity observed in the lower concentration of reagent. These effects can be interpreted by postulating that hydrophobic interactions play an important role in the stability of the original complex while enhanced hydrogen bonding capacity may well be responsible for the

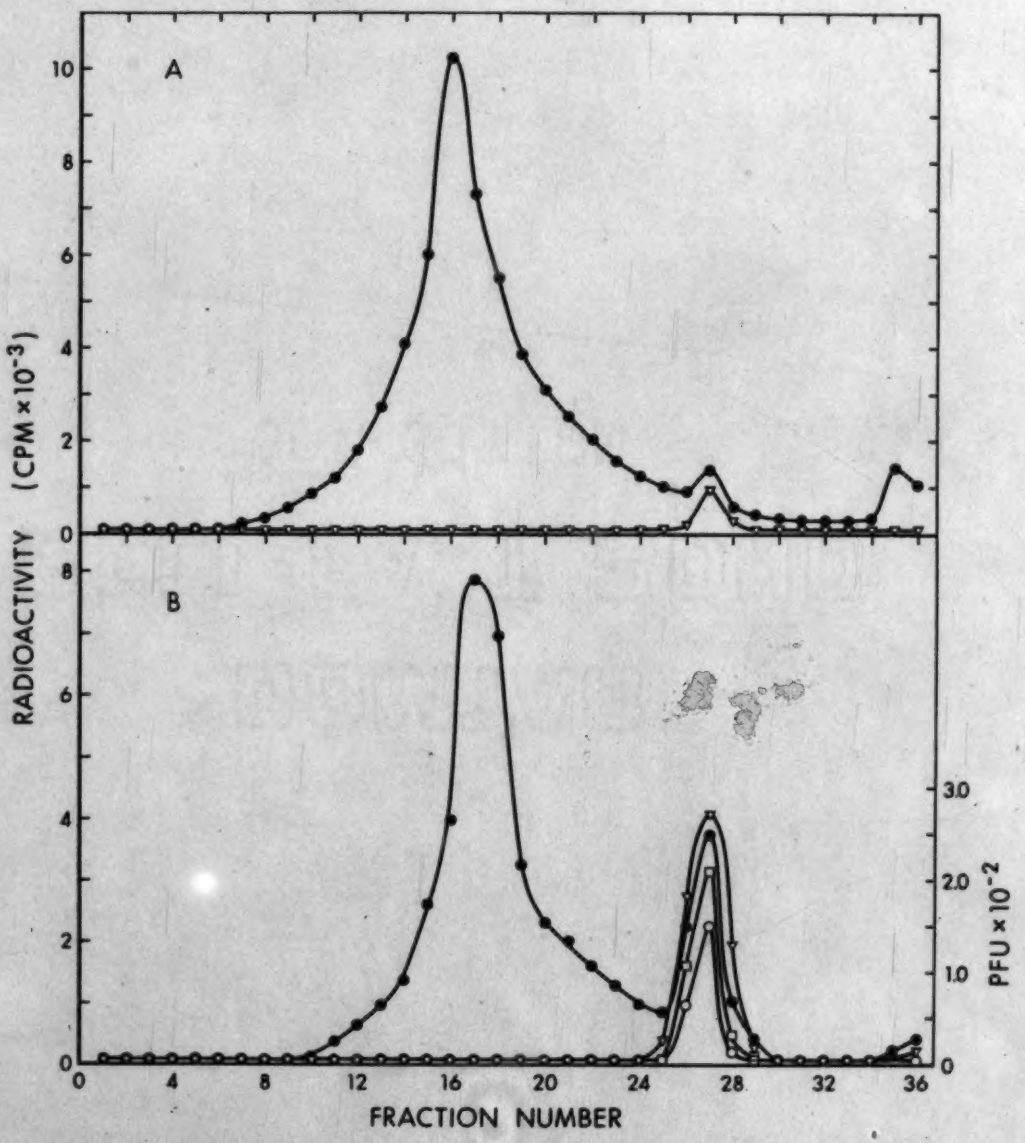


Fig. 4.2 Cesium sulfate density gradient analysis of the acetic acid pellet prepared from a mixture of ^{3}H-histidine labeled and ^{32}P-labeled phage which was incubated in (A) 5% ethylene glycol and (B) 10% ethylene glycol prior to centrifugation. ●——● Cold TCA-insoluble ^{32}P cpm, ▽——▽ Hot TCA-insoluble ^{3}H cpm, □——□ Protein-bound RNA by Nirenberg assay, ○——○ Infectivity (PFU).

formation of the new complex. In 10% ethylene glycol, the new complex contains about 5 moles of A protein per mole of RNA. Since a low level of infectivity (1×10^{-11} PFU per original infectious unit) is associated with the complex, it must contain at least a small number of A protein and RNA molecules orientated in such a way that the infectious process is not impeded.

3. Dissociation of the A Protein-RNA Complex in DMSO

DMSO is an aprotic solvent which can disrupt hydrogen bonds in proteins by displacing bound water and forming stronger hydrogen bonds than water (Henderson et al., 1969). DMSO has also been shown to facilitate the denaturation of nucleic acids (Strauss et al., 1968; Katz and Penman, 1966) presumably by a similar mechanism. Therefore, if hydrogen bonding plays a prominent role in stabilizing the A protein-RNA complex, it would be expected that DMSO would dissociate such a complex. Dissociation of the complex indeed occurs when the pellet is dissolved in either 10% DMSO (Fig. 4.3) or 5% DMSO (not shown) prior to centrifugation and only a single peak containing RNA is observed in the gradient. No hot TCA-insoluble ^{3}H counts appear in any of the fractions but they can be quantitatively recovered from the cellulose nitrate tube.

4. Stability of the A Protein in Intact Particles in Urea and Guanidine-HCl (G-HCl)

The stability of the A protein in the intact virion was investigated in urea and G-HCl. The precise mechanism of these denaturants has not been clarified. Because of their obvious hydrogen bonding potential, it was originally assumed that these agents worked by

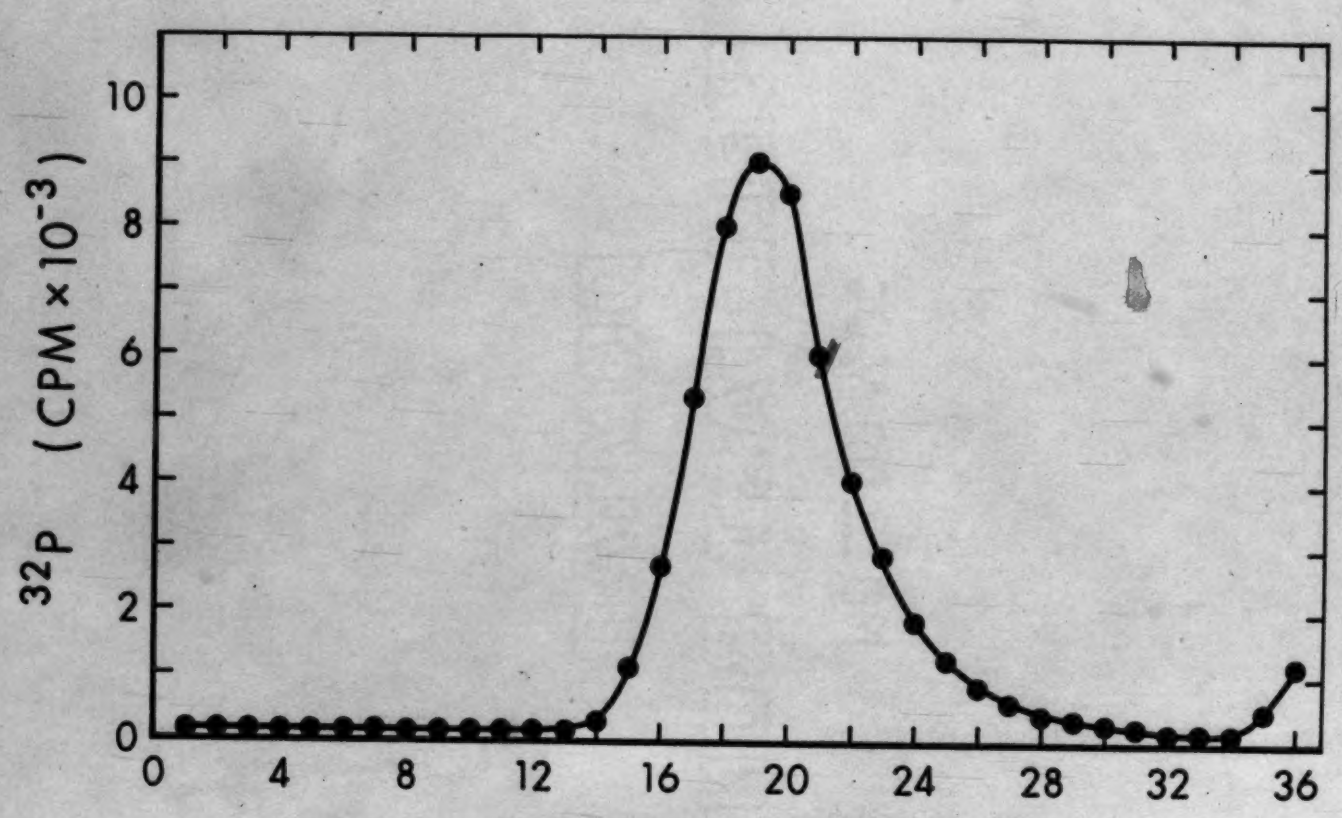


Fig. 4.3 Cesium sulfate density gradient analysis of the acetic acid pellet prepared from a mixture of ^{3}H-histidine labeled and ^{32}P-labeled phage which was incubated in 10% dimethyl sulfoxide prior to centrifugation. ●——● Cold TCA-insoluble ^{32}P cpm.

competing more successfully than water for the donors and acceptors involved in the intramolecular hydrogen bonding in the native macromolecule (Mirsky and Pauling, 1936). This assumption, while not incorrect, must be modified by the finding that the solubility of non-polar substances is generally increased in solutions containing high concentrations of these compounds (Nozaki and Tanford, 1963), and it is now believed that a major part of the denaturing activity of these agents resides in their ability to disrupt hydrophobic interactions. In addition, G-HCl is a strong electrolyte and therefore, electrostatic interactions are minimal in high concentrations of this salt (Tanford, 1968). Indeed, the versatility of G-HCl as a denaturant permits its use in the isolation of RNA (Cox, 1968) and DNA (Pramanick et al., 1976) from nucleoprotein complexes. A concentration of 4 M G-HCl is usually employed in these isolation procedures and it seemed reasonable that this reagent at lower concentration might be a useful probe to test the stability of the link between the A protein and the intact particle.

The sucrose gradient profile of a mixture of ^{32}P-labeled and ^{3}H-histidine labeled R17, which has been disrupted with 8 M urea is shown in Fig. 4.4. It can be seen that the A protein is completely dissociated from the RNA and that the latter sediments at a position characteristic of RNA isolated from R17 by phenolisation. This suggests that hydrogen and/or hydrophobic bonds are required to maintain the association between the A protein and the rest of the particle.

A similar dissociation of A protein from the phage particle was observed in 3 M G-HCl. Fig. 4.5 shows the profile obtained when a

Fig. 4.4 Sucrose gradient sedimentation analysis of urea-treated phage. A mixture of ^{32}P-labeled and ^{3}H-histidine labeled phage was treated with 8 M urea. A 0.2 ml sample of the urea-phage mixture was layered onto a 5 - 20% sucrose gradient containing 8 M urea and centrifugation was carried out as described in Materials and Methods. The arrow indicates the position of phenol-extracted RNA in a parallel gradient. ●——● Cold TCA-insoluble ^{32}P cpm, ▽——▽ Hot TCA-insoluble ^{3}H cpm.

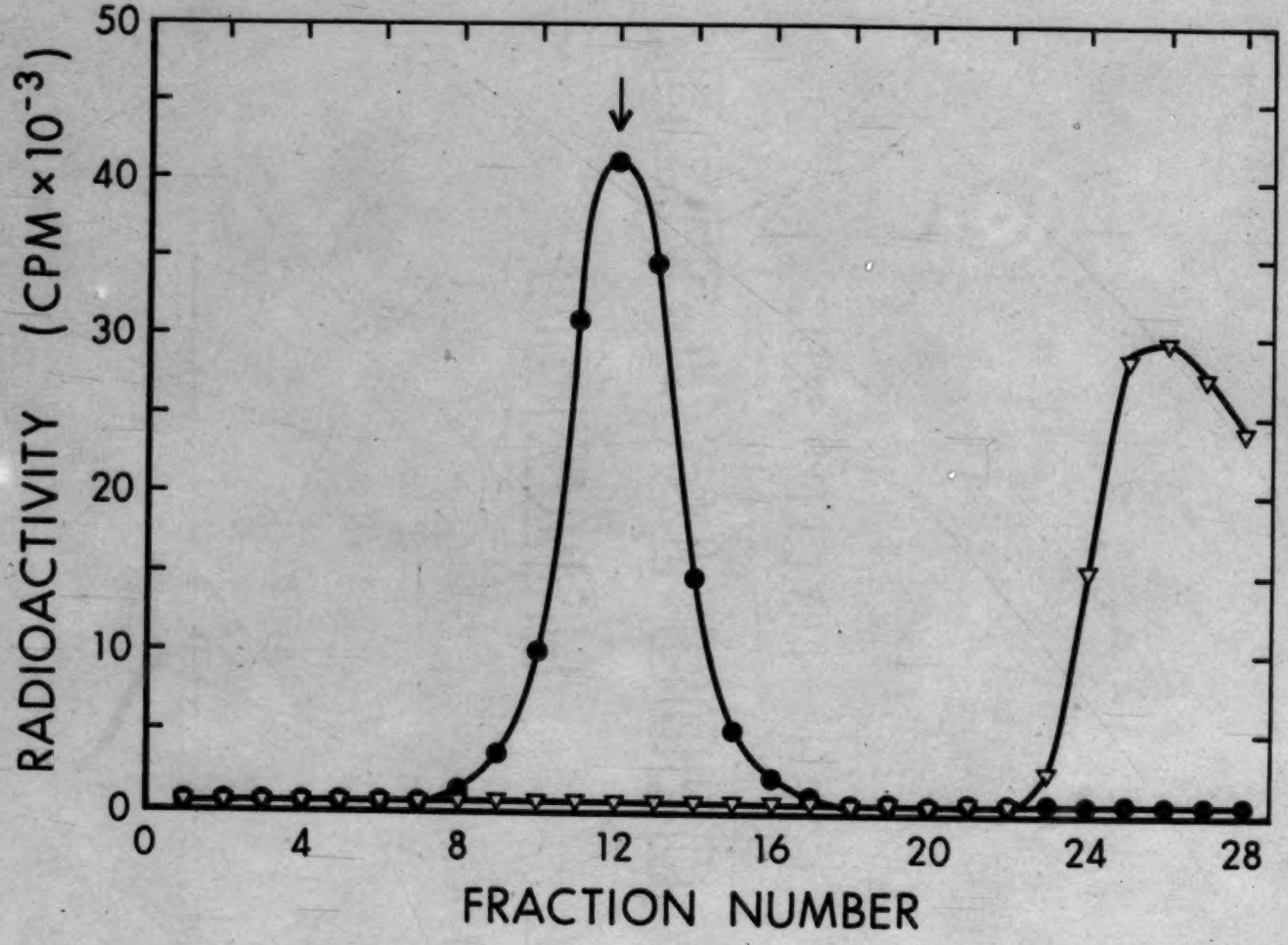
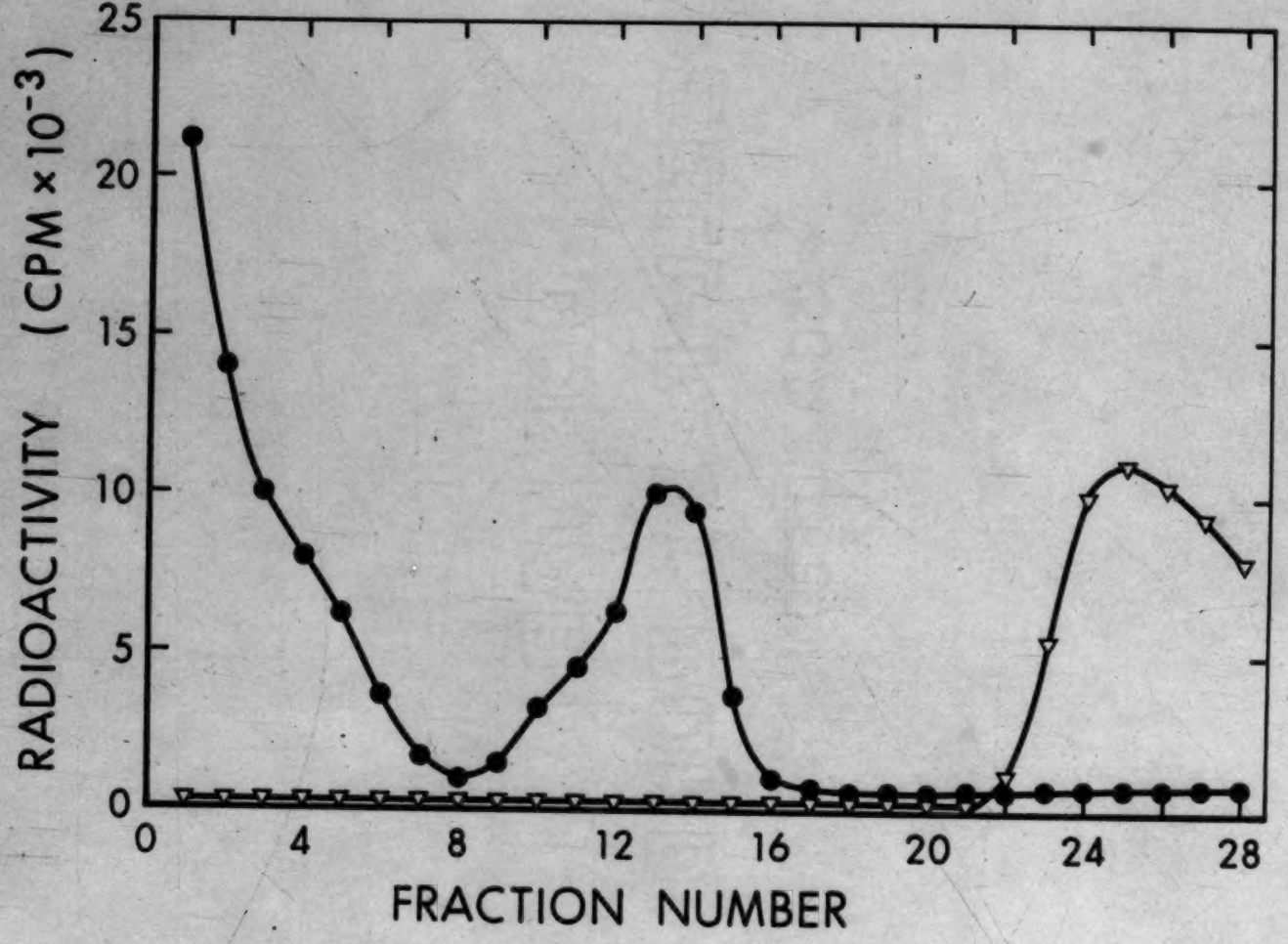

RADIOACTIVITY (CPM ×10⁻³)
50
40
30
20
10
0
0
4
8
12
16
20
24
28
FRACTION NUMBER

Fig. 4.5 Sucrose gradient sedimentation analysis of guanidine hydrochloride-treated phage. A mixture of ^{32}P-labeled and ^{3}H-histidine labeled phage was dialyzed against G-HCl to a final G-HCl concentration of 3 M. A 0.2 ml sample was layered onto a 5 - 20% sucrose gradient containing 3 M G-HCl and centrifugation was carried out as described in Materials and Methods. ●——● Cold TCA-insoluble ^{32}P cpm, ▽——▽ Hot TCA-insoluble ^{3}H cpm.


RADIOACTIVITY (CPM ×10⁻³)
25
20
15
10
5
0
0
4
8
12
16
20
24
28
FRACTION NUMBER

mixture of ^{32}P-labeled and ^{3}H-histidine labeled R17 is dialyzed to 3 M G-HCl at 4° and then subjected to sucrose gradient centrifugation. The material at the bottom of the gradient is probably a mixture of intact and modified particles while the central peak may contain both RNA and more extensively modified particles.

C. Discussion

Since infectious material is clearly present in the isolated A protein-RNA complex, it seems probable that the A protein is linked to the RNA in a manner similar to that presumed to exist during the normal infectious process. It has been suggested by Verbraeken and Fiers (1972) that the attachment of the A protein to the rest of the particle involves ionic interactions since in high concentrations of CsCl, intact virions suffer a progressive loss of A protein. That Cs_2SO_4 might, nonetheless, be a suitable medium for the isolation of an A protein-RNA complex was suggested firstly by the fact that RNA remains sufficiently hydrated to form bands in a solution of this salt, and secondly by the observation that the structural integrity of ribosomes is maintained to a much greater extent in Cs_2SO_4 than in CsCl (De Filippes, 1965). The greater hydration of solute in Cs_2SO_4 solution presumably confers better protection to electrostatic interactions against salt dissociation. Indeed, the stabilizing effect of the SO_4^{2-} anion relative to the Cl^- anion on macromolecular conformation has been well documented (von Hippel and Schleich, 1969).

In an effort to ascertain whether or not hydrophobic and/or

hydrogen bonds contribute to the maintenance of stability in either the A protein-RNA complex or in the bonding of the A protein to the intact particle, use was made of the following perturbants: ethylene glycol, DMSO, urea and guanidine-HCl. Although the data obtained from the use of such agents must be interpreted with caution since few perturbants can be classified as "pure" affectors of any one type of interaction, it seems certain from these studies that ionic interactions are not the only forces involved in stabilization of the A protein-RNA complex. The disruption of the complex in solutions containing ethylene glycol and DMSO implies the existence of hydrophobic and hydrogen bonding, respectively, between the A protein and the RNA, while the removal of the A protein from intact particles in 8 M urea implies a role for these bonding types in the intact particle, also. No deductions can be made with regard to the nature of the bonding disrupted by G-HCl since this agent can potentially affect all three types of interactions.

A detailed study of the sequential degradation of R17 when incubated in 3 M G-HCl for varying lengths of time was reported by O'Callaghan et al., (1973). It was shown that treatment with this reagent at 37° for 10 min yields three modified particle forms of which two are highly unstable in high concentrations of CsCl (as indicated by the loss of RNA during equilibrium centrifugation) and are completely devoid of infectivity. These findings suggest that treatment of intact particles with G-HCl results in a considerable weakening of the link joining the A protein to the capsid and this view is substantiated by the findings in the present study that the

A protein is completely dissociated from the RNA and the rest of the particle in 3 M G-HCl (Fig.4.5) and is partially removed from the particle in 1 M and 2 M guanidine-HCl (not shown).

The isolation of an infectious A protein-RNA complex clarifies, to some extent, the function of the A protein in RNA phages. If such a complex is indeed required for the infectivity of intact phage, then presumably the A protein would have a unique location on the RNA molecule. As discussed in the introductory chapter, Wong and Paranchych (1976a) provide evidence which suggests that the 3'-end of the RNA is the pilot end during infection by intact phage and this led to their proposal that the A protein is complexed to the 3'-end of the genome. If this proposal is correct, then the 3'-end of the RNA must contain a specific region capable of binding the A protein. Such specificity may reside in the untranslated segment [which has a length of 174 nucleotides (Fiers et al., 1976)] at the 3'-terminus of the genome. Also, it is possible that the terminal trinucleotide CCA_{OH} is a required element for this specificity, much in the manner that tRNAs terminating in CCA_{OH} are recognized by amino acyl synthetases. Clearly, further investigations are required both to determine the specificity of the A protein-RNA complex and to elucidate the manner in which the A protein facilitates the transfer of the RNA into the cell.

CHAPTER V

SUMMARY AND DISCUSSION

From the foregoing studies, it seems clear that an A protein-RNA complex is the smallest subviral species of RNA phage which is capable of infecting intact E. coli cells. However, the A protein is presumably required only for the adsorption of phage to the F pilus and for the transport of the RNA into the cell, since naked RNA is infectious in a protoplast system. [An infectivity of 10^{-8} PFU per R17 RNA molecule has been observed by Paranchych (1963) and of 7.5×10^{-8} to 4×10^{-7} PFU per R17 RNA molecule by Argetsinger and Gussin (1966). It is noteworthy that, by addition of protamine sulfate to the protoplast stock and incubation of the phage RNA at 37° prior to protoplast infection, Paranchych was able to increase the infectivity yield to 1 to 2 infectious RNA units per 10^5 phage.] A surface location for the A protein is the most logical one to fulfil its roles as the attachment organelle and pilot protein and the studies described in this thesis verify such a location.

The fate of the A protein during phage infection is poorly understood. Krahn et al. (1972) showed that the interaction of phage particles with F piliated bacteria at 37° leads to the cleavage of the A protein (MW 40,000) into two polypeptides of molecular weights 24,000 and 15,000 which are transferred into the cell along with the phage RNA. The fact that the A protein and RNA are injected in approximately equimolar amounts and that their kinetics of penetration is similar, argues strongly in favor of the idea that the RNA is transferred into the cell as an A protein-RNA complex rather than as free RNA. While it is clear that the A protein is cleaved prior to penetration into the cell, the molecular

mechanism of this cleavage is unknown; nor is it known whether one or both of the A protein cleavage products are required to ensure transport of the RNA into the cell. It has been suggested by Paranchych (1975) that cleavage of the A protein may act as the mechanism which triggers RNA ejection from the phage. Once released from the phage, the A protein and RNA are transported via the pilus into the host cell. It was suggested by Brinton (1971) that the A protein and RNA are transferred into the cell by threading through a hollow core in the F pilus. However, the recent finding of Wong and Paranchych (1976b) that the ejected RNA retains some of its secondary structure, argues against this model since the presence of folded regions in the RNA would confer a greater cross-sectional area on the molecule, thereby precluding its passage through the pilus. Wong and Paranchych (1976b) proposed therefore that the A protein and RNA remain anchored to the exterior of the pilus and are transported into the cell either by a pilus retraction (Marvin and Hohn, 1969; Novotny and Fives-Taylor, 1974) or by a sliding filament (Brinton, 1971) mechanism.

As a means of obtaining information regarding the fate of the A protein and the RNA during early infection, Wong and Paranchych (1976c) monitored the degree of penetration of these phage components during infection by intact phage in the presence of RNase. It was found that penetration of the A protein is unaffected by RNase concentrations as high as 20 μg/ml while the penetration of RNA is greatly reduced at RNase concentrations as low as 0.1 μg/ml. In addition, these studies showed that all of the RNA which enters the cell in the presence of RNase is infectious and intact. This suggests (a) that the RNA on certain sites on the F pilus is protected against nucleolytic attack and (b) that the A protein

can penetrate the cell independently of the RNA. In a similar series of studies, Wong and Paranchych (1976b) showed that in infections carried out with phage whose RNA had been degraded in situ by ascorbate-Cu^{++} treatment, the amount of A protein penetration again remains at the level achieved by untreated phage during the normal infectious process and the amount of RNA penetration is again drastically reduced. However, in this case, examination of the penetrated RNA revealed extensively degraded material. It is therefore evident that RNA penetration is not confined to intact infectious RNA, but that fragments can penetrate as well. These findings are consistent with the view that the A protein acts as the pilot protein which can guide an RNA fragment of varying length into the cell.

From this brief discussion, it is evident that further studies are required to expand our understanding of the role played by the A protein during early infection. While it is now apparent that the A protein functions as an attachment organelle which facilitates the entry of the RNA into the cell, neither the nature of the interaction between the A protein and the pilus nor the means whereby the A protein is translocated along the pilus is known. Another nebulous area concerns the mechanism of cleavage of the A protein and the ultimate fate of the cleavage products after injection into the cell. Clearly, the answers to these questions await future investigations.

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